REMARKS

In the Office Action dated September 2, 2004, the Examiner has entered Applicants' amendment of June 21, 2004, acknowledged Applicants' election with traverse of the claims of Group II for initial prosecution, made the restriction requirement issued on May 20, 2004, final, rejected claims 7-15, 19, and 22-24 as allegedly indefinite under 35 U.S.C.§112, second paragraph, and rejected claims 7-24 for alleged lack of enablement under 35 U.S.C.§112, first paragraph, and under the doctrine of obviousness-type double patenting. The claims are not rejected over the prior art.

Applicants and their representative appreciated the opportunity to discuss this case with the Examiner on December 9, 2004, and thank the Examiner for his time. Applicants believe that the amendments to the claims submitted with this response address the pending indefiniteness and enablement rejections, and place the claims in condition for allowance.

In this response, Applicants cancel claims 7-24 and add new claims 25-40 to more particularly define the claimed methods, and to separately claim aspects of the methods that were previously presented in a single claim. Support for these amendments is found in the original claims and throughout the specification, including at page 7, lines 18-21 (therapeutically effective amount); page 8, line 32 to page 9, line 11 (nucleotides encoding BMP-11); page 12, line 26 to page 13, line 4 (BMP-11 polypeptides in general); page 19, line 28 through page 20, line 12 (administration of BMP-11 to stem cells and neural cells); page 33, lines 10-24 (mature BMP-11 polypeptides); and page 42, Table II (therapeutically effective amount). Upon entry of this amendment, claims 25-40 will be pending.

New claims 25-40 are drawn to methods of promoting survival, inducing neurite formation, inducing differentiation, and modulating proliferation of or from cells. The Examiner has stated that "neuronal cells are neuronal tissue in the absence of evidence to the contrary." Applicants expressly dispute this characterization of neuronal cells and tissues as identical. *Merriam-Webster's Collegiate Dictionary, Tenth Edition*, Springfield, MA (2001) defines tissue as "an aggregate of cells usually of a particular kind together with their intercellular substance that form one of the structural materials of a plant or an animal" (page 1233, copy enclosed). Thus, neuronal tissue includes, but is not identical to, neuronal cells. However, in order to speed prosecution, and without acquiescing to the Examiner's definition of neuronal cells and tissue, Applicants submit new claims 25-40 drawn to methods of administering BMP-11 for effects on cells.

Indefiniteness Rejections under 35 U.S.C.§112, second paragraph

The Examiner has rejected claims 7-16, 19, and 22-24 under 35 U.S.C.§112, second paragraph, for allegedly failing to particularly point out and distinctly claim the subject matter that Applicants regard as the invention. The Examiner alleges that the term "BMP-11" renders claims 7,10, 13, 16, 19, and 22 indefinite, and that claims 7-15 and 22-24 are indefinite for allegedly failing to recite "any result, or a process for producing a result." Further, the Examiner states that the metes and bounds of claims 22-24 are indefinite, alleging that "it is unclear to whom or what the compound is being administered."

First, Applicants believe that one skilled in the art would appreciate the metes and bounds of the "BMP-11 polypeptides" claimed, in view of the description of BMP-11

in the specification, including the portions of application cited by the Examiner. Nevertheless, and without acquiescing to this rejection, Applicants amend the claims to define BMP-11 polypeptides by reference to a sequence identifier to specify the BMP-11 polypeptides within the scope of the pending claims. BMP-11 activity in an osteoinduction assay is described throughout the specification (see, e.g. page 2, lines 20-22), and exemplary osteoinduction assays are set forth in Examples 4-6 (pages 38-43) and were well known in the art at the time of filing. As "BMP-11" is now defined by reference to the human BMP-11 nucleic acid sequence of SEQ ID NO:10 in all claims, the indefiniteness rejection should be withdrawn.

Applicants have met the Examiner's concern that claims 10-15 "lack a process step which clearly relates back to the claim preamble," by amending the new independent claims to add a step that mirrors the claim preamble. Applicants therefore ask that this rejection be withdrawn.

To address the Examiner's concern that the metes and bounds of claims 22-24 are indefinite because it is allegedly "unclear to whom or what the compound is being administered," Applicants note that those claims are canceled, and respectfully suggest that this rejection is now moot.

In view of the foregoing, Applicants respectfully submit that the amended claims are not indefinite, and therefore request that these rejections be reconsidered and withdrawn.

Enablement Rejections under 35 U.S.C.§112, first paragraph

The Examiner has rejected claims 7-24 under 35 U.S.C.§112, first paragraph, as allegedly not enabled by the specification as filed. The Examiner acknowledges that the

specification enables a method of promoting neuronal cell survival, but alleges that the specification does not reasonably provide enablement for a method of modulating or inducing neuronal cell or tissue development, formation, growth, differentiation, proliferation, or maintenance. The Examiner also cites two articles, Schubert et al., *Nature* 344:868-870 (1990) (Schubert) and Jordan et al., *Eur. J. of Neuroscience* 9:1699-1710 (1999) (Jordan), to support an allegation that the skilled artisan would not expect that a BMP, or BMP-11 in particular, would successfully modulate or induce development, formation, growth, differentiation, proliferation, and/or maintenance of a neuronal cell or tissue.

The test of enablement is whether one skilled in the art could make and use the claimed invention from the disclosure coupled with information known in the art without undue experimentation. *United States v. Telectronics, Inc.* 857 F.2d 778, 785 (Fed. Cir. 1988). The test for enablement is not whether any experimentation is necessary, but whether, if experimentation is necessary, it is undue. *In re Angstadt*, 190 USPQ 214 (CCPA 1976), emphasis added. A disclosure may set forth procedures in general terms, and the lack of routine production details does not make a disclosure non-enabling, since one of skill in the art would readily know such details. *Koito Mfg. Co., Ltd. v. Turn-Key-Tech, LLC*, 381 F.3d 1142, 1156 (Fed. Cir. 2004).

The Examiner has the initial burden of giving reasons, supported by the record as a whole, why the specification is not enabling. *In re Angstadt, supra*. The Examiner has not met this burden here. Although the Examiner may have demonstrated that some experimentation is necessary, doing so is not enough to shift the burden to Applicants to prove that such experimentation is not undue. *Id.* The Examiner has not

shown that undue experimentation would be required to practice the claimed invention (MPEP. §2164.01) nor that a reasonable basis exists to question the enablement of the claimed invention (MPEP §2164.04). Even if the evidence relied on by Examiner were enough to shift the burden to Applicants, the evidence presented by Applicants in support of their position clearly outweighs the evidence on which the Examiner relies for his rejection.

As described above, Applicants amend the claims to separately claim aspects of the previously pending claims. Applicants respectfully submit that new claims 25-40 are fully enabled by the specification as filed and that neither Schubert nor Jordan provides a reasonable basis to question the enablement of the claimed methods.

New independent claims 25 and 26, and their dependent claims, are directed to methods of promoting the survival of neuronal cells. These claims are added to support the use of additional forms of BMP-11 in the methods of related U.S. Patent No. 6,340,668. Applicants respectfully submit that these claims are enabled in view of the Examiner's acknowledgement that the specification is enabling for a method of promoting neuronal cell survival. See Office Action at p. 5, lines 18-19. See also, U.S. Patent No. 6,340,668, which issued with claims to methods of promoting the survival of neuronal cells.

New independent claims 27 and 28 are directed to methods of inducing neurite formation from a neural progenitor cell in a mammal and in vitro, respectively. Applicants submit that the specification as filed teaches the skilled artisan how to practice these methods. For example, the application discloses that administration of BMP-11 induces neurite outgrowth in the PC12 model for neurotrophic factors (growth

factors or cytokines affecting the nervous system) (page 46, lines 1-3). Enabling support includes Example 9, which teaches methods of inducing neurite formation in vitro (pages 45, line 29 to page 46, line 3) and stimulating the production of axons and dendrites in mammals (page 19, lines 23-27, page 20, lines 13-30, and page 46, lines 4-22), for example. The specification also sets forth well known means such as microscopy to detect changes in neural cell morphology in response to administration of BMP-11 (see page 46, lines 2-3). Thus, the skilled artisan would have the necessary knowledge to make and use BMP-11 for inducing neurite formation, based on the disclosure of the specification as filed.

New independent claims 29 and 30, directed to methods of inducing differentiation into a neuronal cell in a mammal and in vitro, respectively, are also adequately enabled. As the Examiner acknowledges, the specification discloses "that BMP-11 induces neurite formation in PC12 cells and therefore induces neuronal differentiation." Example 9 teaches a method of inducing differentiation of neuronal cells in vitro comprising administering a composition comprising BMP-11 (pages 45-46, bridge paragraph). Example 9 also discloses assays for promoting differentiation in animal models (page 46, lines 4-22) that were well known in the art and could be applied by a skilled artisan without undue experimentation. Additional guidance on methods for inducing differentiation of neuronal cells can be found at page 18, lines 25-31. Examples of in vivo methods include inducing neuronal regeneration in vivo with agents that affect endogenous stem cells (page 18, lines 27-28), while in vitro methods include cell replacement therapy with ex vivo manipulation of neural stem cells (page 18, lines 28-29). The specification also teaches that BMP-11 can be used to promote

the differentiation of stem cells into neuronal cells and to stimulate the production of axons and dendrites on both neurons that have not developed neurites, as well as neurons that have lost these processes (page 19, lines 23-27). These examples, although stated in general terms, are enabling in view of the level of knowledge of a skilled artisan and the specification as a whole.

Gamer et al., *Developmental Biology* 208:222-232 (1999) (copy enclosed) provides further evidence that the claimed methods for inducing neuronal cell differentiation are fully enabled. Gamer shows that administration of BMP-11 to Xenopus ectodermal explants induces differentiation of neural tissue (Figure 3D), whereas control ectodermal explants differentiate into atypical epidermis (Figure 3C). Thus, Gamer shows that administering BMP-11 protein induces differentiation of progenitor cells to neural cells and tissue as demonstrated by morphological and histological analysis. Further, Figure 4A shows that BMP-11 induces expression of the pan-neural marker, NCAM across a wide range of BMP-11 dosages. These results demonstrate that the claimed methods of inducing neuronal cell differentiation by administering a purified BMP-11 polypeptide of claims 29 and 30 can be employed as set forth in the specification.

Liu et al., *Neuron* 32:997-1012 (2001) (copy enclosed) also provides evidence that the skilled artisan could indeed practice the claimed methods of inducing differentiation of neuronal cells without undue experimentation based on the disclosure of this application. Liu et al. shows that administration of BMP-11 (called GDF-11 in the study) induces the differentiation of motor neurons in an embryonic model of neural patterning (Figure 6F and page 1004, second column). In light of these articles and the

arguments presented above, Applicants submit that the methods for inducing differentiation of neuronal cells of claims 29 and 30 are enabled by the specification as filed.

As claims to "neuronal cell formation" have been canceled, the Examiner's objection that the pending claims allegedly encompass "the formation of neuronal cells from non-neuronal tissues, such as liver, kidney, lung, spleen, or intestine" is moot.

New claims 31 and 32 are directed to methods of modulating proliferation of neuronal cells. Example 9 teaches a method of assessing the number of living cells in neuronal tissue culture (page 45, lines 8-27), and provides data showing that administration of BMP-11 leads to higher numbers of living cells relative to controls (page 45). The skilled artisan would understand from Table II that BMP-11 can be used to practice the method for increasing proliferation of neuronal tissues of claim 33. The assay for living cells taught in Example 9 can also be used to assess a decrease in proliferation. Coupled with the disclosure that administration of BMP-11 to cells in culture induces differentiation (page 45, line 35 to page 46, line 3), the skilled artisan would understand the practicability of the method for decreasing proliferation of neuronal cells of claim 34, and the full scope of the method for modulating proliferation of neuronal cells of claim 31 is enabled. Accordingly, Applicants submit that claims 31-34 are enabled by the specification as filed.

To support the broad allegation that the skilled artisan would not expect that any and/or all BMPs, or BMP-11 in particular, to modulate or induce any and/or all neuronal cell/tissue development, formation, growth, differentiation, proliferation or maintenance,

the Examiner cites and Schubert and Jordan. The Examiner also suggests that these references support the premise that "the effects of BMPs are unpredictable."

Applicants respectfully disagree with the Examiner's characterization of Schubert. The Examiner contends that Schubert teaches "that activin did not increase the survival of ciliary ganglion cells or of several glial cell lines," and therefore that "Schubert does not indicate that any and/or all BMPs would successfully modulate or induce any and/or all neuronal cell/tissue development, formation, growth, differentiation, proliferation, or maintenance." Applicants respectfully submit that activin is not the same protein as BMP-11, nor is activin even a member of the bone morphogenic protein family.[1] On the contrary, as shown in Figure 1 of Massague, *Cell* 69: 1067-70 (1992) (enclosed), activin is a member of a separate protein family, the inhibin/activin family. Thus, the skilled artisan would not expect the effects of BMP-11 to be identical (or even similar) to those of activin. In fact, Schubert does not teach the effects of <u>any</u> BMP protein and thus, does not in any way affect the expectations of the skilled artisan regarding the effects of BMP-11 in the methods of the amended claims. Schubert is cited in the priority application merely for its teaching of how to carry out cell survival assays--not any teaching on the function of BMP-11, or any other BMP.

Applicants respectfully submit that to the extent that Schubert's study of activin A is at all applicable to an enablement analysis of BMP-11, it supports enablement of the

---

1 The priority application refers to BMP-11 as "Activin WC" (see specification at page 1, lines 22-23). The designation was changed in later applications in this series to reflect the classification of BMP-11 within the bone morphogenetic subfamily of the TGF-β superfamily of cytokines (see, for example Jordan at p. 1699) Schubert refers not to BMP-11/Activin WC, but to activin, a distinct protein belonging to a distinct family of TGF- β-like proteins.

scope of the claims. Schubert shows that a protein's lack of effect in a neuronal cell type does not preclude a skilled artisan from understanding that the protein positively affects nerve cells in general. Figure 1 shows that activin A promotes survival of P19, B33 and retinal cells. While stating that activin A did not "increase[ ] the survival of ciliary ganglion cells in the standard assay, nor did [it] promote the survival of several glial, myoblast, or fibrolast cell lines," this peer reviewed article concludes that "[t]he above results show that . . . this protein [activin A] promotes nerve cell survival" (page 870, right column). This is also clear from the title of the Schubert article, "Activin is a nerve cell survival factor." Thus, reading Schubert, one of skill in the art would not question the practicability of the use of activin to promote neural cell survival.

Moreover, Schubert developed a new model for assaying neurotrophic factors, and compared this model to well known biological assays for neurotrophic factors using routine experimentation. As a result, the skilled artisan would know how to confirm the operability of the claimed methods using BMP-11 on various cell types with only routine experimentation in well known assays for neurotrophic effects, including those of Schubert.

Applicants have demonstrated that BMP-11 affects neuronal survival, differentiation, and proliferation, as well as neurite formation in a well accepted model for neurotrophic factors. As described above, subsequent studies using BMP-11 in other assays for neurotrophic factors demonstrate its function in additional progenitor and neural cell types. Techniques to administer and assess the neurotropic effects, for example, of a novel neurologically active agent were well known, and given the disclosure as a whole, a skilled artisan could make and use the claimed invention

across it's full scope. Indeed, Schubert demonstrates a variety of readily available assays by which the skilled artisan could practice the invention with minimal experimentation. Knowing how to routinely test the claimed method in a variety of cell types, the skilled artisan would not question the enablement of the claimed methods over alleged evidence of an inoperable embodiment of a different growth factor—activin A.

Applicants also respectfully disagree with the Examiner's interpretation of Jordan. The Examiner cites three passages in Jordan as alleged evidence that the effects of BMPs are unpredictable, and then concludes that the skilled artisan would not expect that BMP-11 or other BMPs would successfully modulate or induce the neuronal cells and/or tissues as claimed. Applicants respectfully submit that these passages do not meet the burden of establishing that the specification is not enabling for the scope of the pending claims.

The Examiner contends that Jordan teaches that "BMPs 9 and 11 did not promote the in vitro survival of dopaminergic neurons" and that "BMP-11 had no effect on BrdU incorporation and astroglial cell maturation." For at least three reasons, Applicants respectfully submit that these teachings do not provide the skilled artisan with a reasonable basis to question enablement of the claimed methods, which are directed to neuronal outcomes.

First, Applicants respectfully submit that, in fact, Jordan does not contradict Applicants' data showing that BMP-11 increases cell number relative to controls. Jordan shows that administration of a BMP-11 protein at 10 ng/ml to rat midbrain cells in culture for eight days increased the number of dopaminergic neurons to

approximately 130% of controls (See, Figure 4 at page 1702). The 30% increase detected was not statistically significant in Jordan's analysis, which performed three cell counts for two experiments (see Figure 4, legend). Jordan therefore states that "BMPs 9 and 11 did not promote the in vitro survival of dopaminergic neurons," which is understood by the skilled artisan to mean that the effect shown in Figure 4 was not statistically significant.

As amended, the pending claims recite use of a therapeutically effective amount of a BMP-11 polypeptide. Jordan only tests a single dosage (10 ng/ml) of BMP-11 protein, which one skilled in the art would consider a low dose. See, Gamer at 226, right column, first full paragraph. The skilled artisan would understand that the range of concentrations tested in Gamer, for example, which is 10 ng/ml – 1000 ng/ml, is a more reasonable series of conditions to detect an effect of BMP-11. A similar concentration range (0 ng/ml – 1000 ng/ml) is disclosed in Table II on page 42 of the specification of this application, for example, as a range of concentrations for comparing BMP-2 activity to BMP-11. Further, Gamer shows that the skilled artisan could determine effective concentrations of BMP-11 with only routine experimentation.

Second, Jordan describes the failure to observe a statistically significant positive effect of BMP-11 in their assay. One skilled in the art would understand that such negative results can be explained by a variety of technical considerations which may, or may not relate to a biological activity of BMP-11. In contrast, Example 9 demonstrates an approximately 5-fold effect on the number of viable PC12 cells after 6 days of BMP-11 administration at 200 ng/ml. Thus, without disparaging Jordan's BMP-11 results, the skilled artisan would give more weight to the positive data disclosed in Applicants'

specification in assessing the ability of BMP-11 to function in the neuronal outcomes of the claimed methods.

Third, even if Jordan proved conclusively that one or more cell types were not affected by administration of BMP-11 (which Applicants dispute as negative results in view of the single low dose administered), such an inoperable embodiment would not prevent the skilled artisan from understanding that the claimed methods are practicable. Example 9, Gamer, and Schubert demonstrate a variety of dosages and assays by which the skilled artisan could practice the claimed methods with only routine experimentation. As discussed above, Schubert also proves that the skilled artisan would still characterize a protein as "a nerve cell survival molecule" (page 868, title) despite a negative result with a particular biological assay.

The Examiner also cites Jordan as alleged evidence that "not all members of the BMP family share effects on proliferation and differentiation of cells in the astrocyte lineage," and that BMPs are distinct, heterogeneous, and unpredictable with respect to each other. Applicants respectfully submit that alleged evidence of heterogeneity between different BMPs is irrelevant to enablement of Applicants' claims to methods involving a single BMP. Namely, any unpredictability between different BMPs would not cause the skilled artisan to regard the effects of a single BMP family member, such as BMP-11, as internally unpredictable. In fact, Jordan adopts this understanding. The assays disclosed in Jordan assess the effect of a recombinant human BMP-11 in assays involving murine osteoblast-like cells (W-20) and rat neural cells and tissues, for example. Similarly, Schubert's data relating to activin A include cross-species experiments.

In view of the foregoing remarks, Applicants respectfully submit that the claims as presented are fully enabled by the specification and request that the enablement rejections be withdrawn.

Obviousness-type Double Patenting Rejection

The Examiner rejects claims 7-24 under the doctrine of obviousness-type double patenting, in view of U.S. Patent No. 6340668 and, if necessary, in view of U.S. Patent No. 5,700,911 (Wozney). The Examiner alleges that the intended uses of the pending claims do not patentably distinguish the present claims from those of U.S. Patent No. 6,340,668, which claims in vitro and in vivo methods of promoting the survival of neuronal cells with a BMP-11 protein comprising the amino acid sequence of SEQ ID NO: 2 or SEQ ID NO: 11.

Applicants respectfully traverse this rejection as it relates to the amended claims, but, at this time, request that this rejection be held in abeyance until allowable subject matter is indicated. At that time, Applicants will consider whether or not to file a Terminal Disclaimer.

Conclusion

In view of the foregoing amendments and remarks, Applicants respectfully submit that the claimed invention, as amended, is fully set forth and enabled by the specification. Applicants therefore request withdrawal of these rejections and timely allowance of the pending claims.

Applicants believe that any extension of time required to enter this Reply is accounted for in the accompanying Petition for Extension of Time. However, in the

event of an error, please grant any extensions of time necessary and charge any additional required fees to deposit account 06-0916.

Respectfully submitted,

FINNEGAN, HENDERSON, FARABOW,
GARRETT & DUNNER, L.L.P.

Dated: January 3, 2005

By: 
Leslie A. McDonell
Registration No. 34,872

Attachments:

- **Copy of *Merriam-Webster's Collegiate Dictionary, Tenth Edition* (2001), p.1233**
- **Gamer et al., Developmental Biology 208:222-232 (1999)**
- **Liu et al., Neuron 32:997-1012 (2001)**
- **Massague, Cell 69: 1067-70 (1992)**

·pl in constr [It, pl. of timpano ... nore at TYMPANUM] (ca. 1854) ... ed by one performer in an orc...

› plays the timpani
zin tin] (bef. 12c) 1 : a soft ... rystalline metallic element ... emperatures and that is used ... soft solders and alloys — see ... vessel, or a sheet made of tinpl... netal (as aluminum) b : a ... f tomatoes› — **tin** adj — tin...

over or plate with tin or a tin ...

tinamu] (ca. 1783) : any of a ... rican game birds that have a ... y tail and that produce eggs ...

inplate; broadly : CAN 1c 2 ...

(79) : COLORED, TINGED

adj [L tinctorius, fr. tingere to ... dyeing or staining; also : impa...

tinctura act of dyeing, fr. tin... taic : a substance that colors, ... characteristic quality : CAST; ... active principle or extract ... olution of a medicinal substan...

\ˈtiŋ(k)-chə-riŋ, -shriŋ\ (1616) ... E 2 a : to infuse or instill ... : to imbue with a quality : AF... om›
r; akin to OHG zuntra tinder, ... ry flammable substance adap... at serves to incite or inflame ... 1 a : a metal box for holding ... king a spark b : a highly ... illy explosive place or situati... OHG zint point, tine] (bef. 12c) ... RONG 2 : a pointed branch ...

tin·ing \ˈti-niŋ\ [ME, of Scand ... tjón injury, loss — more at ... it : to become lost
worm, moth] (14c) : any of ... WORM — **tin·e·al** \-nē-əl\ ... ea of the leg] (ca. 1923) : ... perineum ... sitive ear ... hin metal sheeting usu. of ...

it. origin] (1602) : a high-pi... e on a crystal goblet — **ting** ... **ing·ing** \ˈtin-jiŋ\ [ME, fr. ... gein to moisten and prob. to ... ith a slight shade or stain ... or taste 2 : to affect or ...

r suffusing shade or color ... fluence : TOUCH ... \-g(ə-)liŋ\ [ME, alter. of ... nging, stinging, prickling; ... sation 2 : TINKLE — **tingle** ... **ly** \-g(ə-)lē\ adj

a gambler) who pretends to ...
) 1 a : a usu. itinerant men... l mender : BUNGLER 2 ...
(ə-)riŋ\ vi (1592) : to work ... just, or work with something ... IDDLE ~ vt : to repair ... ər\ n
am\ n [prob. fr. the tinkers' ... nimum amount or degree ... ut poetry —James Blish› ... — used for a construction ...

ng \-k(ə-)liŋ\ [ME, freq. of ... : to make or emit a tinkle ... NATE ~ vt 1 : to sound ... to cause to make a tinkle ...

in verse or prose 2 : a ...
inkles : TINKLING
name for the Model T Ford ... arly automobile
er 2 : TINSMITH
inging, tinnitus, fr. tinnire to ... of noise (as a ringing or ... (as a disturbance of the ... be heard only by the one ... 1 : of, abounding in, or ... CHEAP 3 : thin in tone ... **in·ni·ness** \ˈti-nē-nəs\ n
at is a center for compo... the body of such compo...

...plate \ˈtin-ˈplāt\ n (1677) : thin sheet iron or steel coated with tin
...plate vt (1890) : to plate or coat (as a metal sheet) with tin
...pot \ˈtin-ˈpät\ adj (1838) : SMALL-TIME
...sel \ˈtin(t)-səl also ˈtin-zəl\ n [MF estincelle, estancele, etincelle ...ark, glitter, spangle — more at STENCIL] (1538) 1 : a thread, strip, or ...eet of metal, paper, or plastic used to produce a glittering and spar...ling appearance in fabrics, yarns, or decorations 2 : something su...perficially attractive or glamorous but of little real worth ‹disfigured ...no gaudy ~ of rhetoric or declamation —Thomas Jefferson›
...sel adj (1575) 1 : made of or covered with tinsel 2 a : cheaply ...audy : TAWDRY b : SPECIOUS ‹~ promises›
...sel vt **tin·seled** or **tin·selled**; **tin·sel·ing** or **tin·sel·ling** \ˈtin(t)-...liŋ also ˈtin-zə-liŋ\ (1594) 1 : to interweave, overlay, or adorn with ...as if with tinsel 2 : to impart a specious brightness to
...sel·ly \ˈtin(t)-s(ə-)lē also ˈtin-zə-lē\ adj (1811) : TINSEL
...smith \ˈtin-ˌsmith\ n (1812) : a worker who makes or repairs things ...sheet metal (as tinplate) — **tin·smith·ing** \-ˌsmi-thiŋ\ n
...stone \ˈtin-ˌstōn\ n (1602) : CASSITERITE
...t \ˈtint\ n [alter. of earlier tinct, fr. L tinctus act of dyeing, fr. tingere ...tinge] (1717) 1 a : a usu. slight or pale coloration : HUE b : any of ...ious lighter or darker shades of a color : TINGE 2 : a variation of a ...lor produced by adding white to it and characterized by a low satura...n with relatively high lightness 3 : a usu. slight modifying quality or ...aracteristic : TOUCH 4 : a shaded effect in engraving produced by ...e parallel lines close together 5 : a panel of light color serving as ...ckground 6 : dye for the hair — **tint·er** n
...t vt (1791) : to impart or apply a tint to : COLOR
...ting n (ca. 1841) 1 : the act or process of one that tints 2 : the en...aved or colored tint produced by tinting
...tin·nab·u·lary \ˌtin-tə-ˈna-byə-ˌler-ē\ adj [L tintinnabulum bell] (1787) : of, relating to, or characterized by bells or their sounds
...tin·nab·u·la·tion \ˌtin-tə-ˌna-byə-ˈlā-shən\ n [L tintinnabulum bell, ...tintinnare to ring, jingle, fr. tinnire] (1831) 1 : the ringing or sound...of bells 2 : a jingling or tinkling sound as if of bells
...less \ˈtint-ləs\ adj (1789) archaic : having no tints : lacking color
...type \-ˌtīp\ n (1864) : FERROTYPE 1
...ware \ˈtin-ˌwar, -ˌwer\ n (1758) : articles and esp. utensils made of ...plate
...work \-ˌwərk\ n (ca. 1934) : work in tin
...y \ˈtī-nē\ adj **ti·ni·er**; **-est** [alter. of ME tine] (1598) : very small or ...minutive : MINUTE syn see SMALL — **ti·ni·ly** \ˈtī-nᵊl-ē\ adv — **ti·ni-...ness** \ˈtī-nē-nəs\ n
...\ˈtip\ vb **tipped**; **tip·ping** [ME] vt (14c) 1 : OVERTURN, UPSET — ...used with over 2 a : CANT, TILT b : to raise and tilt forward in sa...‹tipped his hat› ~ vi 1 : to become tipped : TOPPLE 2 : LEAN, ...ANT — **tip the scales** 1 : to register weight ‹tips the scales at 285 ...unds› 2 : to shift the balance of power or influence ‹tipped the ...les in favor of a declaration of war —S. F. Bemis›
...n (1673) 1 : the act or an instance of tipping : TILT 2 chiefly Brit : a ...ce for depositing something (as rubbish) by dumping
...vt **tipped**; **tip·ping** [ME tipped having a tip, fr. ⁴tip] (14c) 1 a : to ...nish with a tip b (1) : to cover or adorn the tip of (2) : to blend ...s) for improved appearance by brushing the tips of the hair with ... 2 : to affix (an insert) in a book — often used with in 3 : to re...ve the ends of ‹~ raspberries›
...n [ME; akin to MHG zipf tip, OE tæppa tap — more at TAP] (15c) 1 ...usu. pointed end of something 2 : a small piece or part serving as ...end, cap, or point — **tipped** \ˈtipt\ adj — **on the tip of one's ...ngue** 1 : about to be uttered ‹it was on the tip of my tongue to tell ...exactly what I thought› 2 : just eluding recall
...n [ME tippe; akin to LG tippen to tap] (15c) : a light touch or blow
...vb **tipped**; **tip·ping** vt (1567) 1 : to strike lightly : TAP 2 : to give (a ...ball) a glancing blow ~ vi : TIPTOE
...n [origin unknown] (1567) 1 : a piece of advice or expert or author...itive information 2 : a piece of advance or confidential information ...en by one thought to have access to special or inside sources
...vt **tipped**; **tip·ping** (1883) 1 : to impart a piece of information or ...vice about or to — often used with off 2 chiefly Brit : to mention as ...ely candidate, prospective winner, or profitable investment : TOUT ... **tip one's hand** also **tip one's mitt** : to declare one's intentions or ...eal one's opinions or resources ‹the Justice Department wouldn't ...its hand by saying what its next move would be —Newsweek›
...vb **tipped**; **tip·ping** [perh. fr. ⁵tip] vt (ca. 1610) 1 : GIVE, PRESENT ...to give a gratuity to ~ vi : to bestow a gratuity
...n (1755) : a gift or a sum of money tendered for a service per...formed or anticipated : GRATUITY
...cart \ˈtip-ˌkärt\ n (ca. 1877) : a cart whose body can be tipped on the ...frame to empty its contents
...cat \-ˌkat\ n [⁵tip] (1676) : a game in which one player using a bat ...strikes lightly a tapered wooden peg and as it flies up strikes it again to ...it as far as possible while fielders try to recover it; also : the peg ...in this game
...\ˈtē-(ˌ)pē\ var of TEPEE
...in \ˈtip-ˌin\ n [⁶tip] (1948) : a goal in basketball made by deflecting a ...bound into the basket with the fingertips
...off \ˈtip-ˌȯf\ n [⁸tip] (1901) 1 : WARNING, TIP 2 : a telltale sign
...off n [⁶tip] (1922) : the act or an instance of putting the ball in play ...basketball by a jump ball
...of the iceberg [fr. an iceberg being mostly submerged] (1969) : the ...llest, most obvious, or most superficial manifestation of some phe...nomenon
...per \ˈti-pər\ n (1819) : one that tips
...pet \ˈti-pət\ n [ME tipet] (14c) 1 : a long hanging end of cloth at...tached to a sleeve, cap, or hood 2 : a shoulder cape of fur or cloth of...with hanging ends 3 : a long black scarf worn over the robe by ...glican clergymen during morning and evening prayer
...ple \ˈti-pəl\ vb **tip·pled**; **tip·pling** \-p(ə-)liŋ\ [back-formation fr. ...tippler barkeeper] vi (1560) : to drink liquor esp. by habit or to ex...cess ~ vt : to drink (liquor) esp. continuously in small amounts — **tip-...pler** \-p(ə-)lər\ n
...ple n (1581) : DRINK 1, 2
...ple n [E dial. tipple to tip over, freq. of E ¹tip] (1880) 1 : a place ...where or an apparatus by which cars (as for coal) are loaded or emp...tied 2 : a coal-screening plant

tip·py \ˈti-pē\ adj **tip·pi·er**; **-est** (1886) : liable to tip ‹a ~ boat›
tip·staff \ˈtip-ˌstaf\ n, pl **tip·staves** \-ˌstavz, -ˌstāvz\ [obs. tipstaff staff tipped with metal] (1535) : an officer (as a constable or bailiff) who bears a staff
tip·ster \ˈtip-stər\ n (1862) : one who gives or sells tips esp. for gambling or speculation
tip·sy \ˈtip-sē\ adj **tip·si·er**; **-est** [¹tip + -sy (as in tricksy)] (1577) 1 : unsteady, staggering, or foolish from the effects of liquor : FUDDLED 2 : UNSTEADY, ASKEW ‹a ~ angle› — **tip·si·ly** \-sə-lē\ adv — **tip·si·ness** \-sē-nəs\ n
¹**tip·toe** \ˈtip-ˌtō, -ˈtō\ n (14c) : the position of being balanced on the balls of the feet and toes with the heels raised — usu. used with on; also : the ends of the toes — **on tiptoe** : ALERT, AROUSED ‹the contest of skill that puts one on tiptoe to win —Deerfield (Wisc.) Independent›
²**tiptoe** adv (1592) : on or as if on tiptoe
³**tiptoe** adj (1593) 1 : standing or walking on or as if on tiptoe 2 : CAUTIOUS, STEALTHY
⁴**tiptoe** vi **tip·toed**; **tip·toe·ing** (ca. 1661) 1 : to stand or raise oneself on tiptoe 2 : to walk or proceed quietly or cautiously on or as if on tiptoe
¹**tip–top** \ˈtip-ˈtäp, -ˌtäp\ n [⁴tip + top] (1702) : the highest point
²**tip–top** adj (1722) : EXCELLENT, FIRST-RATE ‹~ working conditions›
³**tip–top** adv (1882) : very well
ti·rade \ˈtī-ˌrād also ti-ˈ\ n [F, shot, tirade, fr. MF, fr. OIt tirata, fr. tirare to draw, shoot] (1802) : a protracted speech usu. marked by intemperate, vituperative, or harshly censorious language
tir·a·mi·su \ˌtir-ə-ˈmē-(ˌ)sü, -ˈmi-; -mē-ˈsü\ n [It tiramisù, fr. tirami sù!, lit., pull me up!] (1982) : a dessert made with ladyfingers, mascarpone, chocolate, and espresso
¹**tire** \ˈtīr\ vb **tired**; **tir·ing** [ME tyren, fr. OE tēorian, tӯrian] vi (bef. 12c) : to become weary ~ vt 1 : to exhaust or greatly decrease the physical strength of : FATIGUE 2 : to wear out the patience of : BORE
syn TIRE, WEARY, FATIGUE, EXHAUST, JADE, FAG mean to make or become unable or unwilling to continue. TIRE implies a draining of one's strength or patience ‹the long ride *tired* us out›. WEARY stresses tiring until one is unable to endure more of the same thing ‹*wearied* of the constant arguing›. FATIGUE suggests causing great lassitude through excessive strain or undue effort ‹*fatigued* by the day's chores›. EXHAUST implies complete draining of strength by hard exertion ‹shoveling the wet, heavy snow *exhausted* him›. JADE suggests the loss of all freshness and eagerness ‹appetites *jaded* by overindulgence›. FAG implies a drooping with fatigue ‹shoppers all *fagged* out by the Christmas rush›.
²**tire** n [ME, short for attire] (14c) 1 obs : ATTIRE 2 archaic : a woman's headband or hair ornament
³**tire** vt **tired**; **tir·ing** (14c) 1 obs : ATTIRE 2 archaic : to adorn (the hair) with an ornament
⁴**tire** n, often attrib [ME, prob. fr. ²tire] (15c) 1 : a metal hoop forming the tread of a wheel 2 : a rubber cushion that fits around a wheel (as of an automobile) and usu. contains compressed air
tired \ˈtīrd\ adj (15c) 1 : drained of strength and energy : fatigued often to the point of exhaustion 2 : obviously worn by hard use : RUNDOWN 3 : TRITE, HACKNEYED — **tired·ly** adv — **tired·ness** n
tire·less \ˈtīr-ləs\ adj (1591) : seemingly incapable of tiring : INDEFATIGABLE — **tire·less·ly** adv — **tire·less·ness** n
Ti·re·si·as \tī-ˈrē-sē-əs, -zē-\ n [L, fr. Gk Teiresias] : a blind seer of Thebes who in one Greek myth is changed into a woman for several years and then changed back to a man
tire·some \ˈtīr-səm\ adj (ca. 1520) : WEARISOME, TEDIOUS — **tire·some·ly** adv — **tire·some·ness** n
tir·ing–house \ˈtī-riŋ-ˌhau̇s\ n [³tire] (1590) : a section of a theater reserved for the actors and used esp. for dressing for stage entrances
tir·ing–room \-ˌrüm, -ˌru̇m\ n [³tire] (1623) : a dressing room esp. in a theater
tirl \ˈtərl\ vb [alter. of ¹trill] vi (ca. 1550) chiefly Scot : to make a rattling sound (as with a door latch) ~ vt, chiefly Scot : TWIRL
tiro chiefly Brit var of TYRO
'tis \ˈtiz, (ˌ)tiz, təz\ [contr.] (15c) : it is
ti·sane \ti-ˈzan, -ˈzän\ n [ME, fr. MF, fr. L ptisana, fr. Gk ptisanē, lit., crushed barley, fr. ptissein to crush — more at PESTLE] (14c) : an infusion (as of dried herbs) used as a beverage or for medicinal effects
Tish·ah–b'Ab \ˈti-shə-ˌbäv, -ˌbȯv\ n [Heb tish'āh bĕ'Ābh ninth in Ab] (ca. 1902) : a Jewish holiday observed with fasting on the 9th of Ab in commemoration of the destruction of the temples at Jerusalem
Tish·ri \ˈtish-rē\ n [Heb tishrī] (ca. 1771) : the 1st month of the civil year or the 7th month of the ecclesiastical year in the Jewish calendar — see MONTH table
tis·sue \ˈti-(ˌ)shü, chiefly Brit ˈtis-(ˌ)yü\ n [ME tissu, a rich fabric, fr. OF, fr. pp. of tistre to weave, fr. L texere — more at TECHNICAL] (1563) 1 a : a fine lightweight often sheer fabric b : MESH, NETWORK, WEB ‹a ~ of lies› 2 : a piece of soft absorbent tissue paper used esp. as a handkerchief or for removing cosmetics 3 : an aggregate of cells usu. of a particular kind together with their intercellular substance that form one of the structural materials of a plant or an animal — **tis·su·ey** \ˈti-shə-wē\ adj
tissue culture n (1912) : the process or technique of making body tissue grow in a culture medium outside the organism; also : a culture of tissue (as epithelium)
tissue fluid n (1900) : a fluid that permeates the spaces between individual cells, that is in osmotic contact with the blood and lymph, and that serves in interstitial transport of nutrients and waste
tissue paper n (1777) : a thin gauzy paper used esp. for protecting something (as by covering or wrapping)
tissue plasminogen activator n (1981) : a clot-dissolving enzyme with an affinity for fibrin that is produced naturally in blood vessel linings and is used in a genetically engineered form to prevent damage to heart muscle following a heart attack — abbr. TPA

Developmental Biology 208, 222–232 (1999)
Article ID dbio.1998.9191, available online at http://www.idealibrary.com on IDEAL®

A Novel BMP Expressed in Developing Mouse Limb, Spinal Cord, and Tail Bud Is a Potent Mesoderm Inducer in *Xenopus* Embryos

Laura W. Gamer,[1] Neil M. Wolfman, Anthony J. Celeste, Gary Hattersley, Rod Hewick, and Vicki Rosen

Genetics Institute, Inc., 87 Cambridge Park Drive, Cambridge, Massachusetts 02140

The bone morphogenetic proteins (BMPs) play critical roles in patterning the early embryo and in the development of many organs and tissues. We have identified a new member of this multifunctional gene family, *BMP-11*, which is most closely related to *GDF-8/myostatin*. During mouse embryogenesis, *BMP-11* is first detected at 9.5 dpc in the tail bud with expression becoming stronger as development proceeds. At 10.0 dpc, *BMP-11* is expressed in the distal and posterior region of the limb bud and later localizes to the mesenchyme between the skeletal elements. *BMP-11* is also expressed in the developing nervous system, in the dorsal root ganglia, and dorsal lateral region of the spinal cord. To assess the biological activity of BMP-11, we tested the protein in the *Xenopus* ectodermal explant (animal cap) assay. BMP-11 induced axial mesodermal tissue (muscle and notochord) in a dose-dependent fashion. At higher concentrations, BMP-11 also induced neural tissue. Interestingly, the activin antagonist, follistatin, but not noggin, an antagonist of BMPs 2 and 4, inhibited BMP-11 activity on animal caps. Our data suggest that in *Xenopus* embryos, BMP-11 acts more like activin, inducing dorsal mesoderm and neural tissue, and less like other family members such as BMPs 2, 4, and 7, which are ventralizing and anti-neuralizing signals. Taken together, these data suggest that during vertebrate embryogenesis, BMP-11 plays a unique role in patterning both mesodermal and neural tissues. © 1999 Academic Press

Key Words: BMP; tailbud; limb; spinal cord; *Xenopus*; follistatin.

INTRODUCTION

Bone morphogenetic proteins (BMPs) were originally discovered based on their ability to induce cartilage and bone formation at ectopic sites in animals (Wozney *et al.*, 1988). As members of the TGF-β superfamily, we know today that BMPs function not only in the skeleton, but also in patterning the early embryo and in the development and differentiation of many organs and tissues. BMPs induce a wide variety of biological responses, including cell proliferation, apoptosis, differentiation, and morphogenesis by signaling through serine-threonine kinase receptors and intracellular effectors known as Smads (reviewed in Hogan, 1996; Whitman, 1998).

Much of our knowledge of the biology of BMP/TGF-β factors comes from analysis of early developmental events in *Xenopus*. For example, in the gastrulating *Xenopus* embryo, different classes of TGF-β superfamily ligands are believed to be involved in patterning of both dorsal and ventral mesoderm. Factors such as activin, Vg-1, and the *Xenopus* nodal-related molecules (Xnrs), have been shown to induce dorsal types of mesoderm such as muscle and notochord (reviewed in Slack, 1994; Harland and Gerhart, 1997). In contrast, BMPs 2, 4, and 7 induce ventral types of mesoderm such as blood (reviewed in Graff, 1997). These BMPs also play a role in patterning ectodermal cells by promoting epidermal differentiation and preventing the formation of neural tissue (Wilson and Hemmati-Brivanlou, 1995). During gastrulation, the dorsal lip or Spemann's organizer of the *Xenopus* embryo provides the critical patterning information described above, to the adjacent mesoderm and overlying ectoderm (reviewed in Harland and Gerhart, 1997; Sasai and DeRobertis, 1997). The activities of the organizer are mediated by a group of secreted molecules that include noggin, chordin, and follistatin, which appear to function as TGF-β/BMP antagonists, blocking ventralizing signals in order to specify dorsal

[1] To whom correspondence and reprint requests should be addressed. Fax: (617) 498-8875. E-mail: lgamer@genetics.com.

0012-1606/99 $30.00

mesoderm and neural tissue (reviewed in Thomsen, 1997). Biochemical studies have shown that noggin and chordin act by binding to BMPs 2 and 4, preventing them from interacting with their receptors (Zimmerman *et al.*, 1996; Piccolo *et al.*, 1996). Follistatin is a potent inhibitor of activin and blocks its signaling by physically sequestering the protein (Nakamura *et al.*, 1990). It has recently been shown that follistatin can also block the activity of BMPs 2, 4, and 7 by forming a trimeric complex with BMP and its receptor (Iemura *et al.*, 1998). These data suggest that during early developmental events, there is not only active patterning by factors like BMPs but also selective inactivation of their signaling pathways by secreted antagonists.

In a search for novel BMP/TGF-β-like molecules, we isolated *BMP-11* by a low stringency screen using a *BMP-7* probe. *BMP-11* is a novel member of the BMP/TGF-β superfamily and is most closely related to *GDF-8/myostatin*, a negative regulator of muscle growth (McPherron *et al.*, 1997). In order to determine the function of *BMP-11*, we characterized its expression pattern during mouse embryogenesis and analyzed BMP-11 protein activity in *Xenopus* embryos. Our data suggest a role for BMP-11 in mesodermal and neural patterning in the vertebrate embryo.

MATERIALS AND METHODS

Isolation of BMP-11. In order to search for novel BMP-like molecules, a probe containing the cysteine-rich mature region (nucleotides 1081–1392) of human *BMP-7* was used to screen a bovine genomic library under reduced stringency conditions (final wash 4× SSC/0.1% SDS at 60°C). Positives from this screen were rescreened with a mixed probe corresponding to the mature regions of human *BMPs 5, 6,* and *7* under high stringency conditions (final wash 0.2× SSC/0.1% SDS at 65°C). Clones which hybridized to the *hBMP7* probe under reduced stringency conditions but not to the h*BMP 5, 6, 7* probe mix at high stringency were further characterized. One of these clones, 7r-30, contained a novel bovine BMP-related sequence designated *BMP-11*. This bovine BMP-related sequence was used to design oligonucleotides, which were used to PCR a human *BMP-11*-specific sequence from a human genomic library (Stratagene, Inc.). The human *BMP-11* sequence was then used as a probe to screen human fetal cDNA and human genomic libraries to derive the full-length sequence of human *BMP-11*. The mature region of human *BMP-11* was then used to screen a mouse genomic library (Stratagene, Inc.) to generate full-length clones and sequence for mouse *BMP-11*. The nucleotide sequences of human *BMP-11* and mouse *BMP-11* have been deposited in the GenBank Sequence database.

Whole mount in situ hybridization. Whole mount *in situ* hybridization was performed as described (Hogan *et al.*, 1994) with minor modifications (Herbert Neuhaus, personal communication). The mouse *BMP-11* probe was a 261-bp fragment derived from the 5′ end of the propeptide region of a *BMP-11* cDNA. Following subcloning into pGEM-3, sense and antisense digoxygenin-labeled riboprobes were produced using SP6 and T7 RNA polymerase.

Whole mount-stained embryos were processed, embedded in paraffin, sectioned at 10 μm, and counterstained with eosin as described (Hogan *et al.*, 1994).

Embryological methods. *Xenopus* eggs were fertilized *in vitro*, and embryos were reared by standard methods (Kay and Peng, 1991). Staging was according to Nieuwkoop and Faber (1967). Animal cap ectoderm was isolated from stage 8–9 blastulae and cultured in 0.5 × MMR and 0.5% bovine serum albumin with or without protein factors until the late gastrula stage. The explants were then transferred to 0.75× NAM for long-term culture. Recombinant human BMP-11, mouse noggin, and human activin A were expressed and produced as previously described (Heine *et al.*, 1987; Schlunegger *et al.*, 1992). Recombinant human FGF-2 was purchased from R&D Systems. Recombinant human follistatin was provided by the NIDDK's National Hormone and Pituitary Program and the NICHD. For histology, animal caps were fixed in Bouin's solution, cleared by 70% ethanol washes, and embedded in paraffin. Explants were serially sectioned at 8 μm and stained with hematoxylin and eosin.

Analysis of RNA by RT-PCR. RNA extraction and RT-PCR analyses were performed as described (Wilson and Melton, 1994) with minor modifications (Amanda Frisch, personal communication). The PCR conditions and sequences of the primers for analysis of Xbra, muscle actin, NCAM, Hox B9, EF-1 α, otx 2, Xtwist, and Mix.1 have been previously described (Hemmati-Brivanlou and Melton, 1994; Wilson and Melton, 1994; Blitz and Cho, 1995; Hopwood *et al.*, 1989; Rosa, 1989).

RESULTS

Cloning of BMP-11

A probe containing the mature region of human *BMP-7* was used to screen a bovine genomic library in order to isolate new BMP-like molecules. Several novel TGF-β/BMP-related sequences were isolated and one was designated *BMP-11*. This bovine *BMP-11* sequence was then used to isolate the human *BMP-11* gene, and mature region sequences from human *BMP-11* were used to clone mouse *BMP-11* (a detailed description of the isolation and characterization of human and mouse *BMP-11* will be described elsewhere). The predicted human BMP-11 protein contains 407 amino acids and displays all the features characteristic of BMP family members including a signal sequence for secretion, an RXXR proteolytic processing site, and a carboxyl terminal region containing the highly conserved pattern of cysteine residues (Fig. 1A). In the C-terminal domain, BMP-11 is more similar to TGF-β and inhibin-β, as it contains nine cysteine residues rather than the seven more commonly seen in BMP proteins. Human and mouse BMP-11 share 99.5% identity over the entire amino acid sequence (data not shown). BMP-11 appears to be most closely related to GDF-8/myostatin, sharing 90% amino acid identity within the carboxyl terminal domain (Fig. 1B). Although BMP-11 is more structurally similar to the inhibin class of TGF-β-related proteins because of its cysteine pattern, it shares equal sequence identity (38–42%) in the mature region to BMP-2 and -4 as it does to inhibin βa, βb, and βc (Fig. 1B).

A

```
MVLAAPLLLGFLLLALELRPRGEAAEGPAAAAAAAAAAAAAGVGGERSSR  50

PAPSVAPEPDGCPVCVWRQHSRELRLESIKSQILSKLRLKEAPNISREVV 100

KQLLPKAPPLQQILDLHDFQGDALQPEDFLEEDEYHATTETVISMAQETD 150

PAVQTDGSPLCCHFHFSPKVMFTKVLKAQLWVYLRPVPRPATVYLQILRL 200

KPLTGEGTAGGGGGGRRHIRIRSLKIELHSRSGHWQSIDFKQVLHSWFRQ 250

PQSNWGIEINAFDPSGTDLAVTSLGPGAEGLHPFMELRVLENTKRSRRNL 300

GLDCDEHSSESRCCRYPLTVDFEAFGWDWIIAPKRYKANYCSGQCEYMFM 350

QKYPHTHLVQQANPRGSAGPCCTPTKMSPINMLYFNDKQQIIYGKIPGMV 400

VDRCGCS    407
```

B

```
                *          **                          *   *
BMP-11      CDEHSSESRCCRYPLTVDFEAFGW-DWIIAPKRYKANYCSGQCEYMF---
GDF-8       CDEHSTESRCCRYPLTVDFEAFGW-DWIIAPKRYKANYCSGECEFVF---
BMP-2       ----------CKRHPLYVDFSDVGWNDWIVAPPGYHAFYCHGECPFPLA--
BMP-4       ----------CRRHSLYVDFSDVGWNDWIVAPPGYQAFYCHGDCPFPLA--
Inh-βA      --CDGKVNICCKKQFFVSFKDIGWNDWIIAPSGYHANYCEGECPSHIAGT
Inh-βB      --CDGRTNLCCRQQFFIDFRLIGWNDWIIAPTGYYGNYCEGSCPAYLAGV
Inh-βC      --CQGGSRMCCRQEFFVDFREIGWHDWIIQPEGYAMNFCIGQCPLHIAGM

                                          * *
BMP-11      --MQKYPHTHL-----VQQANPRGSAGPCCTPTKMSPINMLYFNDKQQII
GDF-8       --LQKYPHTHL-----VHQANPRGSAGPCCTPTKMSPINMLYFNGKEQII
BMP-2       DHLNSTNHAIVQTL--VNSVN-SKIPKACCVPTELSAISMLYLDENEKVV
BMP-4       DHLNSTNHAIVQTL--VNSVN-SSIPKACCVPTELSAISMLYLDEYDKVV
Inh-βA      SGSSLSFHSTVINHYRMRGHSPFANLKSCCVPTKLRPMSMLYYDDGQNII
Inh-βB      PGSASSFHTAVVNQYRMRGLNP-GTVNSCCIPTKLSTMSMLYFDDEYNIV
Inh-βC      PGIAASFHTAVLNLLKANTAAGTTGGGSCCVPTARRPLSLLYYDRDSNIV

                      * *
BMP-11      YGKIPGMVVDRCGCS
GDF-8       YGKIPAMVVDRCGCS
BMP-2       LKNYQDMVVEGCGCR
BMP-4       LKNYQEMVVEGCGCR
Inh-βA      KKDIQNMIVEECGCS
Inh-βB      KRDVPNMIVEECGCA
Inh-βC      KTDIPDMVVEACGCS
```

FIG. 1. Sequence of human BMP-11 and its relationship to other members of the TGF-β/BMP superfamily. (A) Predicted amino acid sequence of human BMP-11. The putative proteolytic cleavage site is underlined. The accession number for human BMP-11 is AF100907. (B) Sequence alignment of the carboxyl-terminal region of human BMP-11, mouse GDF-8, human BMP-2, human BMP-4, human inhibin βA, human inhibin βB, and human inhibin βC. Gaps introduced to optimize the alignment are represented by dashes. The conserved cysteine residues are marked by asterisks.

BMP-11 Expression during Mouse Embryogenesis

In order to analyze the spatial expression pattern of *BMP-11*, we performed whole mount *in situ* hybridization on mouse embryos from 8.5 to 12.5 days postcoitum (dpc) and found *BMP-11* transcripts in three main regions: tail bud, limb, and dorsal neural tissue. *BMP-11* was first detected at low levels at 9.0–9.5 dpc in the tip of the tail (Fig. 2A and data not shown). This structure, known as the tail bud, serves as a continuing source of new mesoderm in the postgastrulation embryo (Schoenwolf, 1977; Tam and Beddington, 1987). At 10.0 dpc, expression of *BMP-11* dramatically increases in the tailbud (Fig. 2B). A strong signal continues to be detected in the caudal-most region of the tail until 12.5 dpc (Figs. 2C, 2D, and 2H). Analysis of

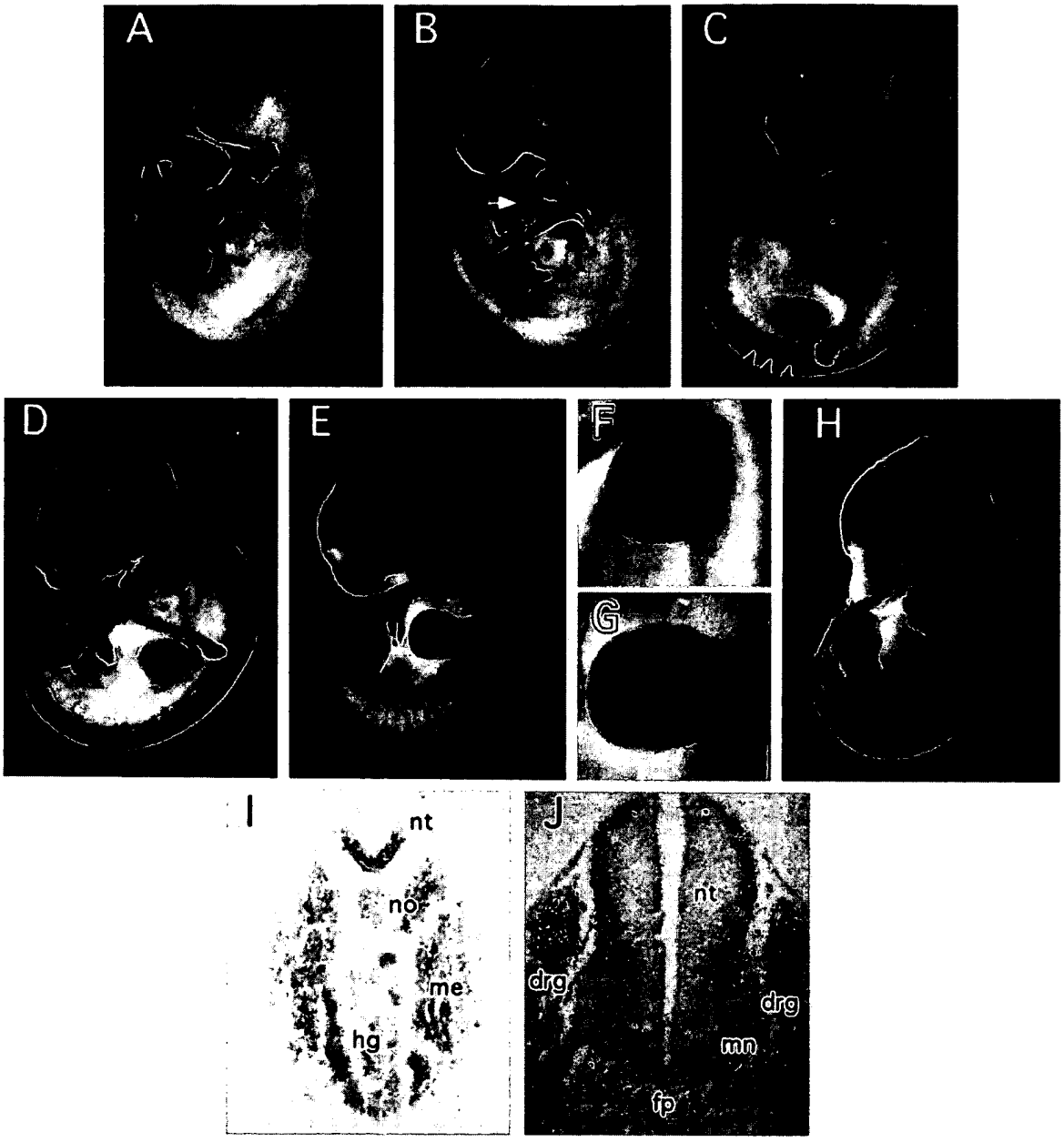

FIG. 2. Whole mount *in situ* hybridization of *BMP-11* expression during mouse embryogenesis. Lateral views of embryos from 9.5 to 12.5 dpc. (A) At 9.5 dpc, weak *BMP-11* expression is seen in the tip of the tail (arrow). (B) At 9.75–10.0 dpc, *BMP-11* expression increases in the tailbud (arrow) and begins to be detected in the developing forelimb bud. (C) At 10.5 dpc, *BMP-11* transcripts are seen in the neural tube and newly differentiated dorsal root ganglia (arrowheads). In the forelimb, *BMP-11* localizes to the distal and posterior mesenchyme. Strong *BMP-11* expression continues in the tailbud. (D) At 11.0 dpc, *BMP-11* expression intensifies in the tailbud, distal limbs, and dorsal root ganglia. (E) At 11.5 dpc, *BMP-11* expression persists in the spinal ganglia, appearing more intense posteriorly, and transcripts in the limbs begin to localize to the mesenchyme surrounding the precartilaginous condensations. (F) High power view of the forelimb at 11.0 dpc showing intense *BMP-11* expression in the distal and posterior regions. No *BMP-11* transcripts are detected in the apical ectodermal ridge. (G) High power view of the forelimb at 11.5 dpc showing BMP-11 expression outlining the newly forming skeletal elements. (H) At 12.5 dpc, *BMP-11* expression is still seen in the spinal ganglia and the caudal most region of the tail. The expression of *BMP-11* in the limb mesenchyme more clearly outlines the forming bones and digits. The apparent signal in the brain, eye, and whisker follicles in whole 11.5–12.5 dpc embryos is due to background associated with older embryos and is seen in the sense strand controls. (I) Transverse section through the tailbud region of 11.0 dpc embryo whole mount *in situ* hybridized with *BMP-11*. Expression is restricted to the neural tube and lateral mesenchyme. (J) Transverse section at a level near the forelimb of an 11.5 dpc embryo whole mount *in situ* hybridized with *BMP-11*. Specific expression is seen in the dorsal lateral region of the developing spinal cord and the dorsal root ganglia. Abbreviations: drg, dorsal root ganglia; fp, floor plate; hg, hindgut; me, mesenchyme; mn, motor neuron; no, notochord; nt, neural tube.

sections through the tail of whole mount-stained embryos at 11.0 dpc reveals specific expression of *BMP-11* in the neuroepithelium and closing neural tube as well as in cells of the dorsal-lateral mesenchyme (Fig. 2I).

The second major area of *BMP-11* expression is in the limbs where expression is initially detected at 10.0 dpc in the distal portion of the forelimb, with the strongest staining in the posterior mesenchyme (Figs. 2B and 2C). From 10.5 to 11.0 dpc, *BMP-11* continues to be expressed at high levels in the distal part of the fore and hind limb, with no signal detected in the apical ectodermal ridge (Figs. 2C, 2D, and 2F). At 11.5 dpc, *BMP-11* expression in the limb mesenchyme begins to outline the precartilaginous condensations, which later differentiate to form bone (Figs. 2E and 2G). *BMP-11* continues to be detected in the mesenchyme between the skeletal elements and is also seen at the digit tips at 12.5 dpc (Fig. 2H).

The third major area of *BMP-11* expression is in the developing nervous system. At 10.5 dpc, *BMP-11* transcripts are detected in the neural tube and the newly forming dorsal root ganglia, which are just beginning to differentiate (Fig. 2C). From 11.0 to 12.5 dpc, *BMP-11* expression is clearly seen in all the dorsal root ganglia, with higher transcript levels detected in the more caudal or newly differentiated spinal primordia (Figs. 2D, 2E, and 2H). Analysis of sections of whole mount embryos at 11.5 dpc revealed specific *BMP-11* expression in the dorsal-lateral edges of the developing spinal cord (excluding the roof plate) and the dorsal root ganglia (Fig. 2J). No expression of *BMP-11* was detected in the ventral neural tube or floor plate. In the dorsal neural tube, the *BMP-11* signal appears to localize to the cell bodies of neurons in the outer mantle layer where sensory relay interneurons develop (Fig. 2J). *In situ* hybridization studies of older embryos (14.0–16.0 dpc) showed that *BMP-11* expression continues in the spinal cord and ganglia, indicating a potential role in later stages of neurogenesis (data not shown).

BMP-11 Induces Morphogenetic Movements in Xenopus Animal Caps

In order to assess the biological activity of BMP-11, we tested the recombinant human protein in the *Xenopus* animal cap assay. Blastula (stage 8) animal pole explants were treated with 200 ng/ml human BMP-11 protein until the gastrula stage (stage 11) and cultured until sibling embryos reached early tailbud tadpole (stage 25). Animal caps cultured in media alone remain round and differentiate into epidermis (Fig. 3A). In contrast, BMP-11 causes a dramatic elongation of animal caps (Fig. 3B). This type of morphology is indicative of mesoderm induction, as the explants try to undergo the movements of gastrulation (Smith *et al.*, 1988). Histological analysis of BMP-11-treated animal caps cultured to late tadpole (stage 38) reveals differentiated blocks of striated muscle, vacuolated notochord cells, and neural tissue (Fig. 3D). Control untreated animal caps form atypical epidermis (Fig. 3C).

Analysis of Gene Expression Induced in Animal Cap Ectoderm by BMP-11

To further assess the kinds of tissues BMP-11 was able to induce, we analyzed gene expression in animal caps treated with various doses of human BMP-11 protein. Animal caps were explanted from late stage 8 embryos and cultured in various concentrations (10 to 1000 ng/ml) of BMP-11 until sibling embryos reached late neurula (stage 22) when they were collected for RT-PCR analysis. We found that BMP-11 induced the expression of the general mesodermal marker, *Xbra*, and the dorsal mesoderm marker, muscle actin, at all doses tested (Fig. 4A). Even at 10 ng/ml (a concentration at which activin, one of the most potent mesoderm inducers, is active), BMP-11 induced *Xbra* and low levels of muscle actin (Fig. 4A). At higher doses (50–1000 ng/ml), BMP-11 induced the pan-neural marker, NCAM (Fig. 4A). This activation of NCAM expression was most likely due to a secondary induction by mesoderm also present in the animal caps (note *Xbra* and muscle actin expression). Our results suggest that in *Xenopus* embryos, BMP-11 is an inducer of dorsal mesoderm and neural tissue.

The mesoderm inducing activity of BMP-11 in the animal cap assay is similar to that of activin but is also comparable to the activity seen for FGF. FGF has been shown to induce mesoderm through a MAP kinase cascade in this system (Gotoh *et al.*, 1995; Umbhauer *et al.*, 1995). Given the recent evidence that BMPs can also activate the MAP kinase pathway (Shibuya *et al.*, 1998), we wanted to determine if BMP-11 was working like activin or FGF to form mesoderm in animal caps. To do this, we treated late blastula stage animal caps with activin (2 ng/ml), FGF (50 ng/ml), and BMP-11 (20 ng/ml) for 2 h (until stage 10.5) and then assayed for the induction of *Mix.1*, a gene that responds to activin, Vg-1, and BMP-4, but not to FGF (Rosa, 1989; Huang *et al.*, 1995; Mead *et al.*, 1996). As expected, activin induced the expression of *Mix.1*, while FGF did not (Fig. 4B). Interestingly, using a relatively low dose of BMP-11, we readily detected the induction of *Mix.1* gene expression (Fig. 4B). These data suggest that in the *Xenopus* embryo, BMP-11 acts more like activin and the TGF-β class of mesoderm inducers.

Follistatin But Not Noggin Inhibits BMP-11 Activity

In *Xenopus* embryos, three secreted factors, noggin, chordin, and follistatin, have been shown to mimic the two main activities of Spemann's organizer: neural induction and dorsalization of mesoderm, by blocking the activities of BMP/TGF-β ligands (reviewed in Harland and Gerhart, 1997; Thomsen, 1997). To determine if any of these antagonists could block BMP-11 activity, we used the animal cap assay. Blastula stage explants were treated with BMP-11 protein (50 ng/ml) alone or with a mixture of BMP-11 (50 ng/ml) and mouse noggin (1000 ng/ml) or human follistatin (500 ng/ml) and cultured until late neurula (stage 22). Noggin, at a 20-fold molar excess, does not block the

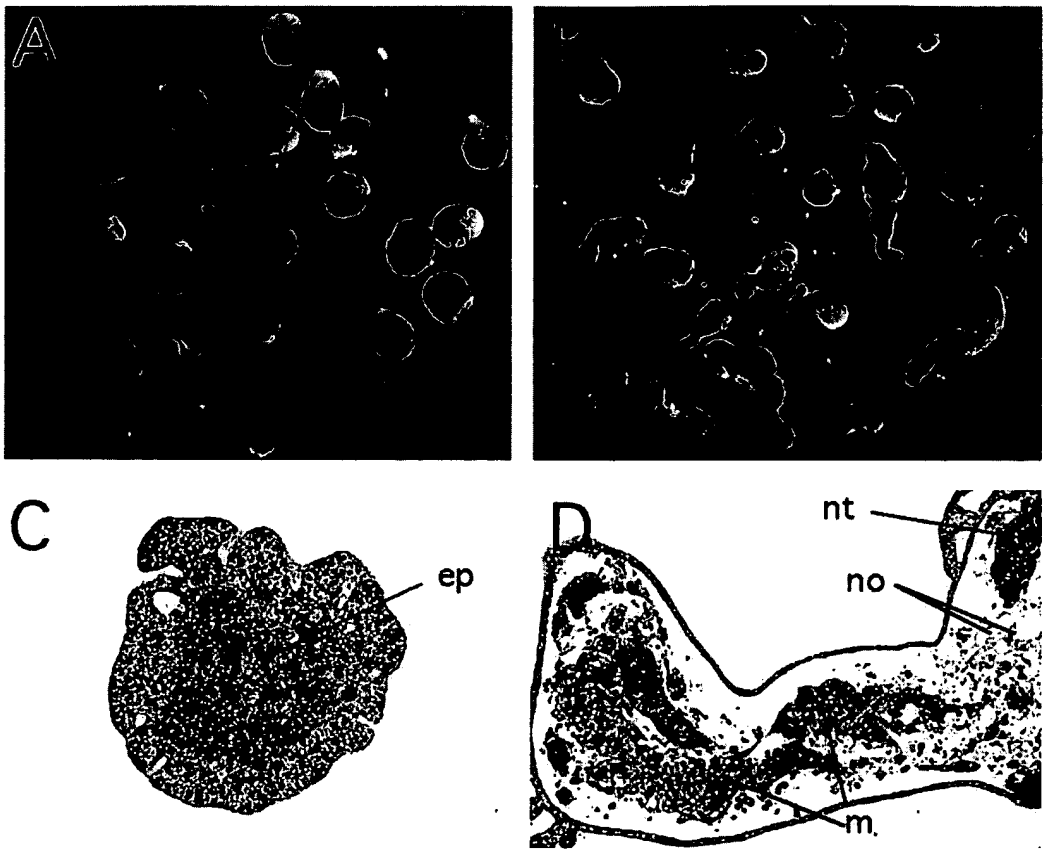

FIG. 3. Morphological and histological analysis of control and BMP-11 treated animal pole explants. Blastula stage (8–9) animal pole explants were treated with 200 ng/ml of recombinant human BMP-11 and cultured to neurula stage for morphology (A, B) and tadpole stage for histology (C, D). (A) Animal caps cultured in media alone remain rounded. (B) Animal caps cultured in BMP-11 protein (200 ng/ml) undergo a dramatic elongation indicative of mesoderm induction. (C) Histological section through a control explant reveals formation of atypical epidermis (ep). (D) Histological section through a BMP-11 treated explant reveals differentiated blocks of muscle (m), notochord (no), and neural tissue (nt).

elongation of animal caps caused by BMP-11, and does not inhibit BMP-11 induction of the dorsal mesoderm marker, muscle actin (Figs. 5B and 5C). The noggin protein used for this assay was active as animal caps incubated in noggin alone at 1000 ng/ml induced the neural marker, NCAM (Fig. 5D). In contrast, follistatin was able to completely inhibit BMP-11-mediated elongation of animal caps at a 10-fold molar excess (Fig. 5F). Moreover, follistatin abolished BMP-11 induction of muscle actin in animal caps (Fig. 5G). In binding experiments, neither noggin nor chordin protein interacted specifically with BMP-11, while follistatin showed significant binding to the BMP-11 protein (L. Fitz and S. Cook, unpublished observations).

BMP-11 Induces Dorsal and Posterior Neural Genes and Modifies the Neural Tissue Induced by Noggin

In *Xenopus*, it is thought that anterior-posterior pattern in the nervous system is induced by the combined action of two signals produced by the dorsal mesoderm (reviewed in Doniach, 1995). The first signal induces anterior neural tissue and can be mimicked by noggin, chordin, and follistatin. The second signal converts the neural tissue induced by the first signal into progressively more posterior types and may reflect the activity of FGFs and Wnts (Cox and Hemmati-Brivanlou, 1995; Lamb and Harland, 1995; McGrew *et al.*, 1995).

At doses from 50 to 1000 ng/ml, BMP-11 induces neural tissue, most likely by a secondary induction through dorsal mesoderm. This raises the question of whether BMP-11 could synergize with neural inducing factors to pattern neural tissue along the anteroposterior axis. In order to address this possibility, we analyzed regional neural marker gene expression in animal cap explants that had been treated with BMP-11 (50 ng/ml), noggin (1000 ng/ml), or a combination of both factors. Animal caps treated with BMP-11 alone induced the expression of the dorsal neural crest marker, *Xtwist* (Hopwood *et al.*, 1989) and the poste-

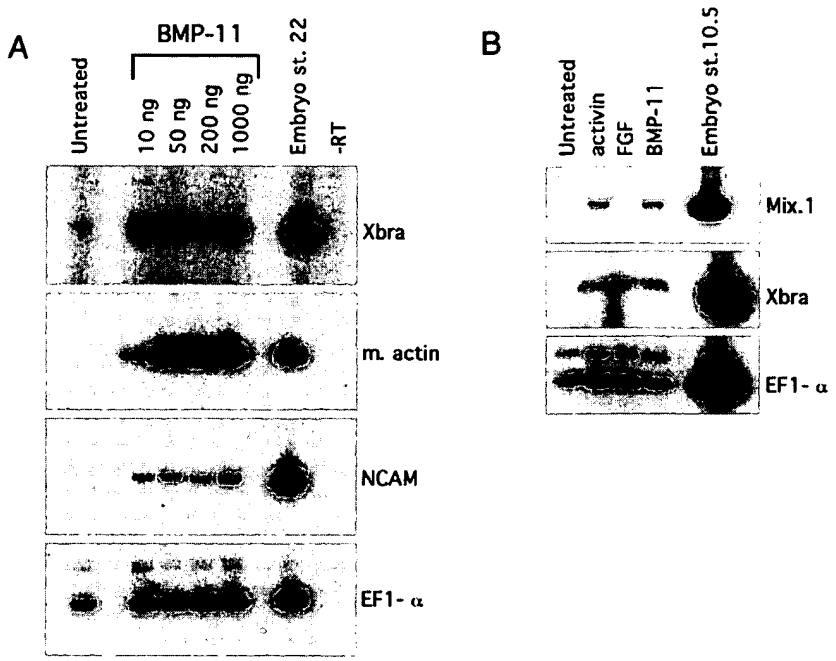

FIG. 4. Analysis of gene expression induced in animal cap explants by BMP-11 protein. (A) Animal caps were explanted from stage 8-9 embryos and treated with increasing doses of recombinant human BMP-11 (10-1000 ng/ml) or buffer alone (untreated) until sibling embryos reached late neurula (stage 22). Total RNA from pools of 10 animal caps was used as the template for cDNA synthesis and the indicated markers were then assayed for by RT-PCR. BMP-11 induced the mesodermal markers *Xbra* and muscle actin (m. actin) at all doses tested. At higher doses (50-1000 ng/ml), BMP-11 induced the pan-neural marker NCAM. EF1-α serves as a loading and reverse transcription control while the embryo and -RT are additional positive and negative controls. (B) Animal caps were explanted from stage 9 embryos and treated for 2 h in buffer alone (untreated), activin (2 ng/ml), FGF (50 ng/ml), or BMP-11 (20 ng/ml) and collected at gastrula (stage 10.5). Low doses of activin and BMP-11 induced the expression of *Mix.1*, while FGF did not. All factors tested induced mesoderm as seen by the expression of *Xbra*.

rior spinal cord marker, *HoxB9* (Wright *et al.*, 1990) (Fig. 6), but not the anterior forebrain marker, *otx2* (Blitz and Cho, 1995). Noggin treated explants only induced anterior neural tissue as detected by *otx2* expression (Lamb *et al.*, 1993) (Fig. 6). Interestingly, animal caps incubated in both BMP-11 and noggin have a reduced level of *otx2* while *Xtwist* and *HoxB9* continue to be expressed (Fig. 6). These results indicate that BMP-11 can modify the neural tissue induced by noggin to more dorsal and posterior fates. This alteration of neural patterning is probably not direct and occurs as a consequence of BMP-11's strong mesoderm-inducing activity.

DISCUSSION

We have been using developmental biology to characterize novel BMP proteins. Here we report the expression pattern and biological activity during vertebrate embryogenesis of *BMP-11*, a new TGF-β superfamily factor that shows high homology to *GDF-8/myostatin*. In the developing mouse, *BMP-11* is expressed in regions of active mesodermal and neural patterning such as the tailbud, limb bud, and dorsal neural tube. In *Xenopus* embryos, BMP-11 is a potent inducer of axial mesoderm (muscle and notochord) and can also induce and modify neural tissue. In addition, BMP-11 activity is inhibited by follistatin but not by noggin or chordin. Taken together our data suggest roles for *BMP-11* in mesodermal formation and neurogenesis in the embryo.

In mouse embryos, *BMP-11* is first detected at low levels in the tip of the tail (tailbud) and as development proceeds, expression dramatically increases and persists until 13.0 dpc. At 9.5 dpc, when we initially see *BMP-11* transcripts in the tail, the tailbud replaces the primitive streak and node as the secondary organizing center of the embryo and new mesoderm continues to arise from this region for several days (Tam and Beddington, 1987; Schoenwolf, 1977). Early and persistent expression of *BMP-11* in the tailbud and its mesoderm inducing ability suggest this factor plays an important role in tail formation and posterior mesodermal patterning. This potential function for *BMP-11* would be similar to that of *Brachyury*, a key regulator of mesodermal cell specification in both mouse and *Xenopus* embryos (Hermann, 1992).

Like many members of the BMP family, *BMP-11* is highly

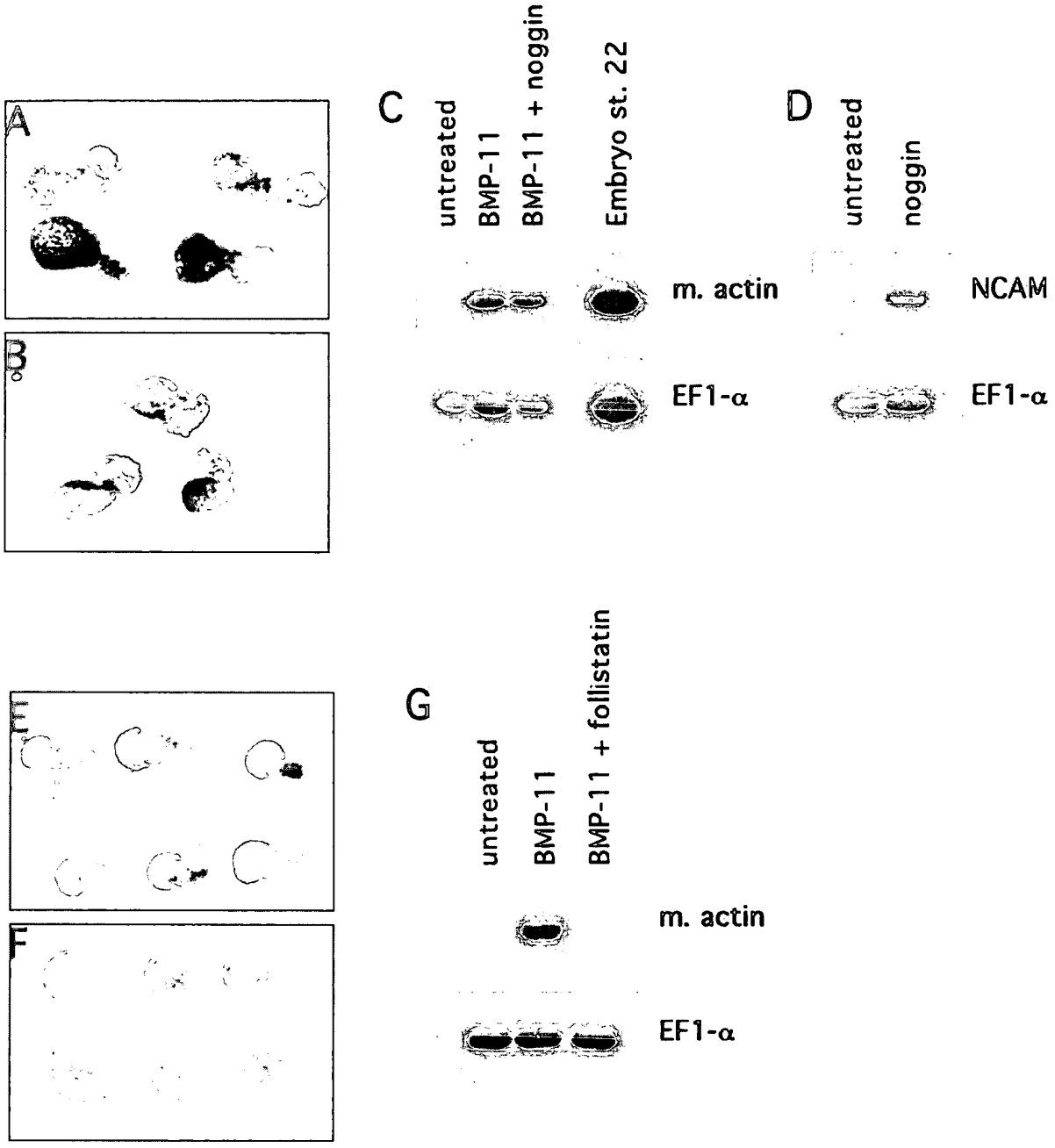

FIG. 5. Follistatin but not noggin inhibits BMP-11 activity. Blastula stage animal pole explants were treated with 50 ng/ml BMP-11 protein alone (A, E) or in combination with 1000 ng/ml mouse noggin protein (B) or 500 ng/ml human follistatin protein (F) until late neurula (stage 22). (A) BMP-11 induces morphogenetic movements in animal caps. (B) A 20-fold molar excess of noggin does not inhibit BMP-11 activity. (C) RT-PCR analysis shows that noggin is unable to block BMP-11 induction of muscle actin (m. actin). (D) Recombinant mouse noggin induces the neural marker, NCAM, indicating the protein has the predicted activity in our assay. (E) BMP-11 protein induces elongation in animal caps. (F) Follistatin completely inhibits the activity of BMP-11 at a 10-fold molar excess. (G) RT-PCR analysis shows that follistatin fully blocks muscle actin induction by BMP-11. EF1-α is a loading and reverse transcription control and untreated animal caps serve as a negative control.

expressed in the developing limb bud, being initially detected in the distal mesenchyme, and later localizing to regions around the developing bones. Initial results from implanting BMP-11 soaked beads in the chick indicate that ectopic BMP-11 causes truncation of skeletal elements in the developing wing (L. Gamer and K. Cox, unpublished

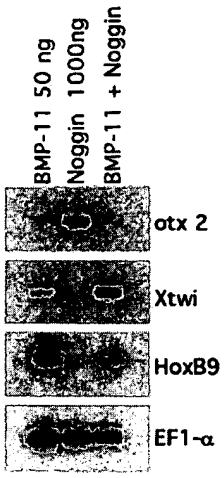

FIG. 6. BMP-11 induces region-specific neural markers and modifies the neural tissue induced by noggin. Blastula stage animal pole explants were treated with BMP-11 (50 ng/ml), noggin (1000 ng/ml), or a combination of both factors until early neurula (stage 18) and analyzed for regional neural marker expression by RT-PCR. BMP-11 induces the neural crest marker, *Xtwist* (Xtwi), and the spinal cord marker, *Hox B9*, but not the forebrain marker, *otx2*. Noggin alone induces only *otx2* expression. In combination, BMP-11 dramatically reduces the amount of *otx2* induced by noggin and allows continued expression of *Xtwi* and *Hox B9*. EF1-α serves as a loading and reverse transcription control.

results). In the context of the limb, *BMP-11* might act as a dual modulator of pattern affecting both mesenchymal cell growth and differentiation. This type of regulatory function is seen with other BMPs such as *BMP-2*, which acts on early undifferentiated chick limb mesenchymal cells to cause apoptosis and then later promotes growth and differentiation of developing cartilage in the limb (Macias *et al.*, 1997).

In the developing nervous system, *BMP-11* is expressed in the neural tube and in the dorsal root ganglia. Dorsal root ganglia are derived from neural crest cells which have already migrated from the dorsal most aspect of the closing neural tube (reviewed in Bronner-Fraser, 1994). Because we do not detect any *BMP-11* expression in this region of the early neural tube, we do not believe *BMP-11* is involved in neural crest cell formation. We do believe, however, that the initial and persistent expression of *BMP-11* in the dorsal root ganglia suggests a later role for this factor in the survival or proliferation of these neurons as they differentiate.

In the neural tube, *BMP-11* localizes to the dorsal lateral edges of the developing spinal cord. Generation of the diverse cell types found in this region of the spinal cord is partially dependent on TGF-β-related signals (reviewed in Tanabe and Jessell, 1996; Bronner-Fraser and Fraser, 1997). For example, *BMP-4* and *BMP-7* are expressed early in the epidermal ectoderm and roof plate and provide dorsalizing signals, which induce neural crest cells and specific subsets of interneurons in developing chick neural tube (Liem *et al.*, 1995, 1997). Because of the later and broader expression of *BMP-11* in dorsal neural tube and its ability to induce and indirectly pattern neural tissue in *Xenopus* embryo explants, we suggest that *BMP-11* may act as a secondary source of BMP signaling, which continues the generation of dorsal cell identity in the developing spinal cord.

The dorsal neural tube is also a rich source of molecules shown to be involved in somitogenesis (for reviews see Currie and Ingham, 1998). In vertebrates, the specification of somitic mesoderm is controlled by factors which are expressed by neighboring tissues. The ventral neural tube and notochord promote formation of the sclerotome, which gives rise to the precursor cells of the ribs, vertebrae, and intervertebral discs. The dorsal neural tube and surface ectoderm promote differentiation of the dermomyotome and myotome, which give rise to dermis and striated muscle. In the chick, BMP-4 has been shown to play a role in dorsal somitic patterning by its ability to block myogenesis in the dermomyotome (Pourquie *et al.*, 1996; Reshef *et al.*, 1998). Based on the above observation, and the expression pattern and activity of *BMP-11*, we suggest that *BMP-11* may also function as a mediator of somite formation, perhaps by modulating sclerotome or dermomyotome differentiation along with other BMPs.

The activity of BMP-11 on *Xenopus* ectodermal explants is most similar to that seen with activin treatment, including induction of dorsal mesoderm and neural tissue. Since BMP-11 is more structurally similar to the inhibin/activin class of TGF-β molecules and because BMP ligands can signal through different receptor combinations (reviewed in Massague, 1998), the activity of BMP-11 could reflect its signaling through an activin type II receptor and downstream activation of the Smad2 pathway. This is most likely the case, as we have found no homologous sequences to *BMP-11* in *Xenopus* by genomic Southern analysis (L. Gamer, unpublished observations). To further investigate this hypothesis, we are currently conducting studies to determine whether the actions of BMP-11 can be blocked by overexpressing dominant negative activin type I and type II receptor constructs in *Xenopus* embryos.

Using the animal cap assay, we also found that BMP-11 activity was specifically blocked by follistatin, but not by noggin. This interaction between BMP-11 and follistatin may be meaningful during development as the expression patterns of both genes localize to adjacent domains in certain regions of the mouse embryo. *BMP-11* is expressed in the dorsal lateral neural tube at a time when follistatin is detected in the adjacent somites (Feijen *et al.*, 1994). In addition, *BMP-11* is expressed around the precartilaginous condensations when follistatin is expressed in those condensing cartilages in the limb (Feijen *et al.*, 1994). Taken together our data suggest that follistatin may be involved in limiting BMP-11 activity during somitogenesis and limb development in a manner similar to the way noggin modifies BMP-4 activity in the somite and limb (McMahon *et al.*, 1998; Reshef *et al.*, 1998; Capdevila and Johnson, 1998).

Recent evidence suggests that follistatin may inhibit BMP activity by a mechanism that is different from noggin and chordin (Iemura *et al.*, 1998). We do not yet know how follistatin antagonizes BMP-11, but binding studies to address this question should be possible upon the identification of a BMP-11 receptor.

BMP-11 is highly related to *GDF-8/myostatin*, a factor recently shown to be an important negative regulator of skeletal muscle mass (McPherron *et al.*, 1997). Interestingly, like other highly related BMPs such as BMP-2 and BMP-4, BMP-11 and GDF-8 proteins appear to have similar activity in the *Xenopus* animal cap assay (L. Gamer, unpublished observations). This suggests that these two factors may be binding to the same or a similar receptor and using an analogous signaling pathway. Although these factors appear to have similar activities, BMP-11 cannot compensate for the loss of GDF-8 in homozygous null animals. This may reflect their mutually exclusive expression domains as *GDF-8* is specifically expressed in developing somites and skeletal muscle (McPherron *et al.*, 1997), where we do not detect any *BMP-11* transcripts. These data suggest that *BMP-11* and *GDF-8* adopted different functions as they evolved from a common ancestral gene.

ACKNOWLEDGMENTS

We thank Amanda Frisch for excellent technical advice with RT-PCR, Diane Peluso for animal cap histology, Jennifer Dube and Adele Pearson for mouse BMP-11 *in situ* probes, and Herbert Neuhaus, Paul Yaworsky, and Lisa-Anne Whittemore for critical reading of the manuscript.

REFERENCES

Blitz, I. L., and Cho, K. W. (1995). Anterior neurectoderm is progressively induced during gastrulation: The role of the *Xenopus* homeobox gene orthodenticle. *Development* **121**, 993–1004.

Bronner-Fraser, M. (1994). Neural crest cell formation and migration in the developing embryo. *FASEB J.* **8**, 699–706.

Bronner-Fraser, M., and Fraser, S. E. (1997). Differentiation of the vertebrate neural tube. *Curr. Opin. Cell Biol.* **9**, 885–891.

Capdevila, J., and Johnson, R. L. (1998). Endogenous and ectopic expression of noggin suggests a conserved mechanism for regulation of BMP function during limb and somite patterning. *Dev. Biol.* **197**, 205–217.

Cox, W. G., and Hemmati-Brivanlou, A. (1995). Caudalization of neural fate by tissue recombination and bFGF. *Development* **121**, 4349–4358.

Currie, P. D., and Ingham, P. W. (1998). The generation and interpretation of positional information within the vertebrate myotome. *Mech. Dev.* **73**, 3–21.

Doniach, T. (1995). Basic FGF as an inducer of anteroposterior neural pattern. *Cell* **83**, 1067–1070.

Feijen, A., Goumans, M. J., and van den Eijnden-van Raaij, A. J. M. (1994). Expression of activin subunits, activin receptors and follistatin in postimplantation mouse embryos suggests specific developmental functions for different activins. *Development* **120**, 3621–3637.

Gotoh, Y., Masuyama, N., Suzuki, A., Ueno, N., and Nishida, E. (1995). Involvement of the MAP kinase cascade in *Xenopus* mesoderm induction. *EMBO J.* **14**, 2491–2498.

Graff, J. M. (1997). Embryonic patterning: To BMP or not to BMP, that is the question. *Cell* **89**, 171–174.

Harland, R. M., and Gerhart, J. C. (1997). Formation and function of Spemann's organizer. *Annu. Rev. Cell Dev. Biol.* **13**, 611–667.

Heine, U. I., Munoz, E. F., Flanders, K. C., Ellingsworth, L. R., Lam, H. Y. P., Thompson, N. L., Roberts, A. B., and Sporn, M. B. (1987). Role of transforming growth factor-β in the development of the mouse embryo. *J. Cell Biol.* **105**, 2861–2876.

Hemmati-Brivanlou, A., and Melton, D. A. (1994). Inhibition of activin receptor signaling promotes neuralization in *Xenopus*. *Cell* **77**, 273–281.

Hermann, B. G. (1992). Action of the Brachyury gene in mouse embryogenesis: Postimplantation development in the mouse. *CIBA Found. Symp.* **165**, 78–91.

Hogan, B., Beddington, R., Costantini, F., and Lacy, E. (1994). "Manipulating the Mouse Embryo," 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

Hogan, B. (1996). Bone morphogenetic proteins: Multifunctional regulators of vertebrate development. *Genes Dev.* **10**, 1580–1594.

Hopwood, N. D., Pluck, A., and Gurdon, J. B. (1989). A *Xenopus* mRNA related to *Drosophila twist* is expressed in response to induction in the mesoderm and the neural crest. *Cell* **59**, 893–903.

Huang, H. C., Murtaugh, L. C., Vize, P. D., and Whitman, M. (1995). Identification of a potential regulator of early transcriptional responses to mesoderm inducers in the frog embryo. *EMBO J.* **23**, 5965–5973.

Iemura, S-I., Yamamoto, T. S., Takagi, C., Uchiyama, H., Natsume, T., Shimasaki, S., Sugino, H., and Ueno, N. (1998). Direct binding of follistatin to a complex of bone-morphogenetic protein and its receptor inhibits ventral and epidermal fates in early *Xenopus* embryo. *Proc. Natl. Acad. Sci. USA* **95**, 9337–9342.

Kay, B. K., and Peng, H. B. (Eds.) (1991). *Xenopus* laevis: Practical uses in cell and molecular biology. *In* "Methods Cell Biology." Academic Press, San Diego.

Lamb, T. M., Knecht, A. K., Smith, W. C., Stachel, S. E., Ecomomides, A. N., Stahl, N., Yancopolous, G. D., and Harland, R. M. (1993). Neural induction by the secreted polypeptide noggin. *Science* **262**, 713–718.

Lamb, T. M., and Harland, R. M. (1995). Fibroblast growth factor is a direct neural inducer, which combined with noggin generated anterior-posterior neural pattern. *Development* **121**, 3627–3636.

Liem, K. F., Tremml, G., Roelink, H., and Jessell, T. M. (1995). Dorsal differentiation of neural plate cells induced by BMP-mediated signals from epidermal ectoderm. *Cell* **82**, 969–979.

Liem, K. F., Tremml, G., and Jessell, T. M. (1997). A role for the roof plate and its resident TGFβ related proteins in neuronal patterning in the dorsal spinal cord. *Cell* **91**, 127–138.

Macias, D., Ganan, Y., Sampath, T. K., Piedra, M. E., Ros, M. A., and Hurle, J. M. (1997). Role of BMP-2 and OP-1 (BMP-7) in programmed cell death and skeletogenesis during chick limb development. *Development* **124**, 1109–1117.

Massague, J. (1998). TGFβ signal transduction. *Annu. Rev. Biochem.* **67**, 753–791.

McGrew, L. L., Lai, C-J., and Moon, R. T. (1995). Specification of the anteroposterior neural axis through synergistic interaction of the Wnt signaling cascade with noggin and follistatin. *Dev. Biol.* **172**, 337–342.

McMahon, J. A., Takada, S., Zimmerman, L. B., Fan, C-M., Harland, R. M., and McMahon, A. P. (1998). Noggin-mediated antagonism of BMP signaling is required for growth and patterning of the neural tube and somite. *Genes Dev.* **12**, 1438–1452.

McPherron, A. C., Lawler, A. M., and Lee, S-J. (1997). Regulation of skeletal muscle mass by a new TGF-β superfamily member. *Nature* **387**, 83–90.

Mead, P. E., Brivanlou, I. H., Kelley, C. M., and Zon, L. I. (1996). BMP-4 responsive regulation of dorsal-ventral patterning by the homeobox protein Mix.1. *Nature* **382**, 357–360.

Nakamura, T., Takio, K., Eto, Y., Shibai, H., Titani, K., and Sugina, H. (1990). Activin binding protein from rat ovary is follistatin. *Science* **247**, 836–838.

Nieuwkoop, P. D., and Faber, J. (1967). "Normal Table of *Xenopus laevis.*" North-Holland, Amsterdam.

Piccolo, S., Sasai, Y., Lu, B., and DeRobertis, E. M. (1996). Dorsoventral patterning in *Xenopus:* inhibition of ventral signals by direct binding of chordin to BMP-4. *Cell* **86**, 589–598.

Pourquie, O., Fan, C. M., Coltey, M., Hirsinger, E., Watanabe, Y., Breant, C., Francis-West, P., Brickell, P., Tessier-Lavigne, M., and LeDouarin, N. M. (1996). Lateral and axial signals involved in avian somite patterning: A role for BMP-4. *Cell* **84**, 461–471.

Reshef, R., Maroto, M., and Lassar, A. B. (1998). Regulation of dorsal somitic cell fates: BMPs and noggin control the timing and pattern of myogenic regulator expression. *Genes Dev.* **12**, 290–303.

Rosa, F. M. (1989). *Mix.1,* a homeobox mRNA inducible by mesoderm inducers, is expressed mostly in the presumptive endodermal cells of *Xenopus* embryos. *Cell* **57**, 965–974.

Sasai, Y., and DeRobertis, E. M. (1997). Ectodermal patterning in vertebrate embryos. *Dev. Biol.* **182**, 5–20.

Schlunegger, M. P., Cerletti, N., Cox, D. A., McMaster, G. K., Schmitz, A., and Grutter, M. G. (1992). Crystallization and preliminary X-ray analysis of recombinant human transforming growth factor β2. *FEBS Lett.* **303**, 91–93.

Schoenwolf, G. C. (1977). Tail (end) bud contributes to the posterior region of the chick embryo. *J. Exp. Zool.* **201**, 227–246.

Slack, J. M. (1994). Inducing factors in *Xenopus* early embryos. *Curr. Biol.* **4**, 116–126.

Smith, J. C., Yaqoob, M., and Symes, K. (1988). Purification, partial characterization and biological effects of the XTC mesoderm inducing factor. *Development* **103**, 591–600.

Tam, P. P. L., and Beddington, R. S. P. (1987). The formation of mesodermal tissues in the mouse embryo during gastrulation and early organogenesis. *Development* **99**, 109–126.

Tanabe, Y., and Jessell, T. (1997). Diversity and patterning in the developing spinal cord. *Science* **274**, 1115–1122.

Thomsen, G. H. (1997). Antagonism within and around the organizer: BMP inhibitors in vertebrate body patterning. *Trends Genet.* **13**, 209–211.

Umbhauer, M., Marshall, C. J., Mason, C. S., Old, R. W., and Smith, J. C. (1995). Mesoderm induction in *Xenopus* caused by activation of MAP kinase. *Nature* **376**, 58–62.

Whitman, M. (1998). Smads and early developmental signaling by the TGFβ superfamily. *Genes Dev.* **12**, 2445–2462.

Wilson, P. A., and Hemmati-Brivanlou, A. (1995). Induction of epidermis and inhibition of neural fate by BMP-4. *Nature* **376**, 331–333.

Wilson, P. A., and Melton, D. A. (1994). Mesodermal patterning by an inducer gradient depends on secondary cell–cell communication. *Curr. Biol.* **4**, 676–686.

Wozney, J. M., Rosen, V., Celeste, A. J., Mitsock, L. M., Whitters, M. J., Kriz, R. W., Hewick, R. M., and Wang, E. M. (1988). Novel regulators of bone formation: Molecular clones and activities. *Science* **242**, 1528–1534.

Wright, C. V. E., Morita, E. A., Wilkin, D. J., and DeRobertis, E. M. (1990). The *Xenopus* X1Hbox6 homeo protein, a marker of posterior neural induction, is expressed in proliferating neurons. *Development* **109**, 225–234.

Zimmerman, L. B., De Jesus-Escobar, J. M., and Harland, R. M. (1996). The Spemann organizer signal noggin binds and inactivates bone morphogenetic protein 4. *Cell* **86**, 599–606.

Received for publication October 23, 1998
Accepted December 23, 1998

Neuron, Vol. 32, 997–1012, December 20, 2001, Copyright ©2001 by Cell Press

Assigning the Positional Identity of Spinal Motor Neurons: Rostrocaudal Patterning of Hox-c Expression by FGFs, Gdf11, and Retinoids

Jeh-Ping Liu,[1,3] Ed Laufer,[2]
and Thomas M. Jessell[1,4]
[1]Howard Hughes Medical Institute
Department of Biochemistry
and Molecular Biophysics
Columbia University
701 West 168th Street
New York, New York 10032
[2]Department of Genetics and Development
Columbia University
701 West 168th Street
New York, New York 10032
[3]Department of Neuroscience
University of Virginia School of Medicine
Lane Road Extended
Charlottesville, Virginia 22908

Summary

Subclasses of motor neurons are generated at different positions along the rostrocaudal axis of the spinal cord. One feature of the rostrocaudal organization of spinal motor neurons is a position-dependent expression of *Hox* genes, but little is known about how this aspect of motor neuron subtype identity is assigned. We have used the expression profile of Hox-c proteins to define the source and identity of patterning signals that impose motor neuron positional identity along the rostrocaudal axis of the spinal cord. We provide evidence that the convergent activities of FGFs, Gdf11, and retinoid signals originating from Hensen's node and paraxial mesoderm establish and refine the Hox-c positional identity of motor neurons in the developing spinal cord.

Introduction

Developing neurons possess positional identities that permit them to form selective connections with target cells. In the vertebrate central nervous system (CNS), neurons acquire their positional identity in response to the spatially restricted actions of extrinsic signaling factors. Typically, these factors act by inducing the expression of transcription factors that impose the identity of their neuronal progeny. In this view, the position that a progenitor cell occupies in the neural tube is a critical determinant of its later neuronal identity. Details of the signaling pathways by which neurons acquire positional identity, however, remain poorly defined.

The steps that link neuronal position and identity have been explored in the developing spinal cord. Here, neuronal patterning appears to be regulated by signaling systems that operate along the dorsoventral (D-V) and rostrocaudal (R-C) axes of the neural tube (Lumsden and Krumlauf, 1996; Tanabe and Jessell, 1996). The D-V pattern of neuronal generation depends on bone morphogenetic protein (BMP) and Hedgehog signaling (Briscoe and Ericson, 2001; Lee and Jessell, 1999). The secretion of Sonic hedgehog (Shh) from the notochord and floor plate is critical for the patterning of ventral cell types (Briscoe and Ericson, 2001; Patten and Placzek, 2000). Shh signaling controls ventral neuronal fates by regulating the expression profile of homeodomain (HD) proteins that determine the positional identity of postmitotic neurons (Briscoe and Ericson, 2001).

The mechanisms that establish the identity of neuronal subtypes along the R-C axis of the spinal cord are less well defined. Interneuron subclasses are typically generated along the entire R-C extent of the spinal cord, whereas developing motor neurons (MNs) exhibit marked R-C differences in identity. Two major distinctions in the R-C identity of spinal MNs have been defined through studies of their position, axon trajectory, and pattern of muscle innervation (Landmesser, 2001). One is the allocation of MNs to discontinuous columnar divisions: thus lateral motor column (LMC) neurons are generated only at limb levels, whereas visceral MNs are generated at thoracic levels (Hollyday, 1980a, 1980b; Landmesser, 1978a, 1978b). A second distinction is evident in the formation of pools of MNs that occupy distinct R-C positions within the LMC, each pool innervating a different target muscle (Hollyday, 1980a; Landmesser, 1978b). These distinctions in the R-C positional identity of MNs have been linked to the expression of transcription factors (Jessell, 2000). Columnar subclasses of MNs can be delineated by the profile of LIM-HD protein expression (Tsuchida et al., 1994), and MN pools can be recognized by the expression of ETS proteins (Lin et al., 1998). Furthermore, genetic studies have begun to provide evidence that LIM-HD and ETS proteins regulate the subtype identity and pattern of connectivity of developing MNs (Sharma et al., 1998; Kania et al., 2000; S. Arber, personal communication).

An additional R-C distinction in MN identity, superimposed upon programs of column and pool organization is evident as a graded positional value that is linked to the topographic projections of somatic and visceral MNs within their target fields (Forehand et al., 1994; Laskowski and Sanes, 1987). The only known molecular correlate of this graded R-C positional identity of MNs is the expression of members of the *Hox* gene family (Belting et al., 1998; Ensini et al., 1998; Lance-Jones et al., 2001). Moreover, there is emerging functional evidence that *Hox* genes control the projection patterns of motor axons. Inactivation of members of the *Hox-c* and *Hox-d* gene clusters leads to alterations in motor innervation of specific muscles in the limb (Carpenter et al., 1997; de la Cruz et al., 1999; Tiret et al., 1998), and in the developing hindbrain, *Hox-a* and *Hox-b* genes control the identity and axonal trajectory of cranial MNs (Bell et al., 1999; Gavalas et al., 1997; Jungbluth et al., 1999; Studer et al., 1996).

Despite evidence for Hox-based R-C positional differences in MN identity, the extrinsic signals that control *Hox* gene expression in the spinal cord have not been defined. Grafting studies in chick embryos have provided evidence that the positional identity of spinal MNs,

[4]Correspondence: tmj1@columbia.edu

as well as the pattern of *Hox* gene expression, can be respecified soon after neural tube closure by signals derived from the paraxial mesoderm (Ensini et al., 1998). Similarly, in the hindbrain, signals from the paraxial mesoderm have been implicated in the regulation of *Hox* gene expression (Grapin-Botton et al., 1997; Itasaki et al., 1996). Nevertheless, it remains unclear whether signals from the paraxial mesoderm are sufficient to establish the R-C positional identity of MNs. In the lumbar spinal cord, for example, the developmental profile of *Hox-d* gene expression cannot easily be accounted for by signals from the paraxial mesoderm (Lance-Jones et al., 2001).

In this study, we have examined the factors that establish the graded R-C positional identity of MNs, through an analysis of Hox-c protein expression. Our findings indicate that the convergent activities of three classes of extrinsic signals—FGFs, Gdf11, and retinoids—appear to be involved in establishing the Hox-c positional identity of spinal MNs.

Results

Rostrocaudal Expression of Hox-c Proteins in the Developing Spinal Cord

To generate markers that define the positional identity of MNs along the R-C axis of the developing spinal cord, we raised antibodies against Hoxc5, Hoxc6, Hoxc8, Hoxc9, and Hoxc10. These antibodies were used to define the temporal and spatial pattern of Hox-c expression in the embryonic chick spinal cord.

Temporal Pattern

The neural expression of Hox-c proteins was first detected at HH stage 14, when low levels of Hoxc8, Hoxc9, and Hoxc10 were expressed in a few cells in the caudal neural tube (data not shown). From HH stages 16 to 22, the peak period of MN generation (Hollyday and Hamburger, 1977), the expression of Hoxc5, Hoxc6, Hoxc8, Hoxc9, and Hoxc10 was restricted primarily to Isl1(2)$^+$, HB9$^+$ MNs (Figure 1A; data not shown). Newly differentiated MNs are located medially, and these neurons lacked Hox-c expression (Figure 1A; data not shown), indicating that the onset of Hox-c expression occurs after MNs have left the cell cycle. Hox-c expression in MNs persisted at HH stage 24, close to the end of MN generation, and at this stage expression was also detected in other cell types (Figure 1C). From HH stages 24 to 28, the expression of Hox-c proteins, notably Hoxc8 and Hoxc9, became restricted to columnar subsets of MNs (Supplemental Figure S1 at http://www.neuron.org/cgi/content/full/32/6/997/DC1).

Spatial Pattern

We next analyzed the pattern of Hox-c expression in MNs at different R-C levels of the spinal cord, from HH stages 16 to 24. Over this period, different Hox-c proteins were expressed in restricted R-C domains. At HH stage 24, Hoxc5 was detected throughout the cervical spinal cord, with a posterior limit of expression in the rostral brachial spinal cord (Figures 1B and 1C). Hoxc6 was detected from caudal cervical levels into the brachial region (Figures 1B and 1C). Hoxc8 was detected from the mid-brachial to the mid-thoracic levels (Figures 1B and 1C). Hoxc9 was detected from caudal brachial through thoracic levels (Figures 1B and 1C). Hoxc10 was detected in the lumbar spinal cord (Figures 1B and 1C). These results extend previous studies (Belting et al., 1998; Ensini et al., 1998), and provide evidence that developing spinal MNs exhibit R-C identities that can be defined by their Hox-c expression profile.

Early Specification of the Neural Pattern of Hox-c Expression

To examine how the R-C identity of spinal MNs is established, we assayed the timing of specification of Hox-c expression in chick neural tissue in vitro. We first analyzed the profile of Hox-c expression in neural explants isolated from different R-C positions of the neural tube in 14 to 15 somite stage (s) embryos. Ventral neural tissue positioned caudal to the most recently formed somites and rostral to Hensen's node (HN) was isolated together with prospective or definitive floor plate cells, to provide a source of Shh to initiate spinal MN generation (Ericson et al., 1996). Neural tissue was subdivided into five R-C domains, each domain was cultured in vitro for 66 hr (to the equivalent of HH stages 24–25), and then assayed for Hox-c expression (Figure 2A). The positional fate of neural cells in these five domains was determined in vivo (see Experimental Procedures).

Cells in neural explants fated to populate caudal cervical levels expressed Hoxc6, but not other Hox-c proteins (Figure 2B). Cells in neural explants fated to give rise to rostral brachial levels expressed Hoxc6 and low levels of Hoxc8, but not Hoxc9 or Hoxc10 (Figure 2B). Neural explants fated to give rise to caudal brachial levels contained many Hoxc6$^+$ and Hoxc8$^+$ cells, a few Hoxc9$^+$ cells, but no Hoxc10$^+$ cells (Figure 2B). Neural explants fated to give rise to rostral thoracic levels expressed fewer Hoxc6$^+$ and many Hoxc8$^+$ and Hoxc9$^+$ cells, but no Hoxc10$^+$ cells (Figure 2B). Cells in neural explants fated to populate caudal thoracic levels of the neural tube expressed low levels of Hoxc6, higher levels of Hoxc8 and Hoxc9, but not Hoxc10 (Figure 2B). These caudal thoracic explants contain Hoxc6$^+$ and Hoxc8$^+$ cells, even though the spinal cord at this level in vivo lacks expression of both proteins (Figures 1B and 1C), an issue we return to below. No expression of Hoxc5 was detected in any of these explants (data not shown). Many Hox-c$^+$ cells were MNs, as assessed by coexpression of Isl1(2), although Hox-c proteins were also detected in other cell types, as in vivo (Figure 2C; data not shown). The expression of each of these Hox-c proteins was first detected between 24 hr–48 hr (data not shown), a timing consistent with their onset of expression in vivo. Thus, neural Hoxc6 to Hoxc10 expression patterns in chick embryos are specified by the 14s stage.

We also monitored the Hox-c profile of MNs generated in neural plate tissue isolated from 5s, 10s, and 20s (HH stages 8+, 10, and 13+) embryos (Figures 3A, 3C, and 3E). Ventral neural tissue located anterior to HN in 5s embryos is fated to populate rostral cervical levels, and none of the MNs generated in these explants expressed Hoxc6, Hoxc8, Hoxc9, or Hoxc10 (Figures 3A and 3B). Ventral neural tissue isolated from the region anterior to HN in 10s embryos is fated to populate rostral brachial levels, and MNs in these explants expressed Hoxc6 and Hoxc8, but not Hoxc9 or Hoxc10 (Figures 3C and 3D; data not shown). Ventral neural tissue isolated from the region anterior to HN in 20s embryos is fated to populate

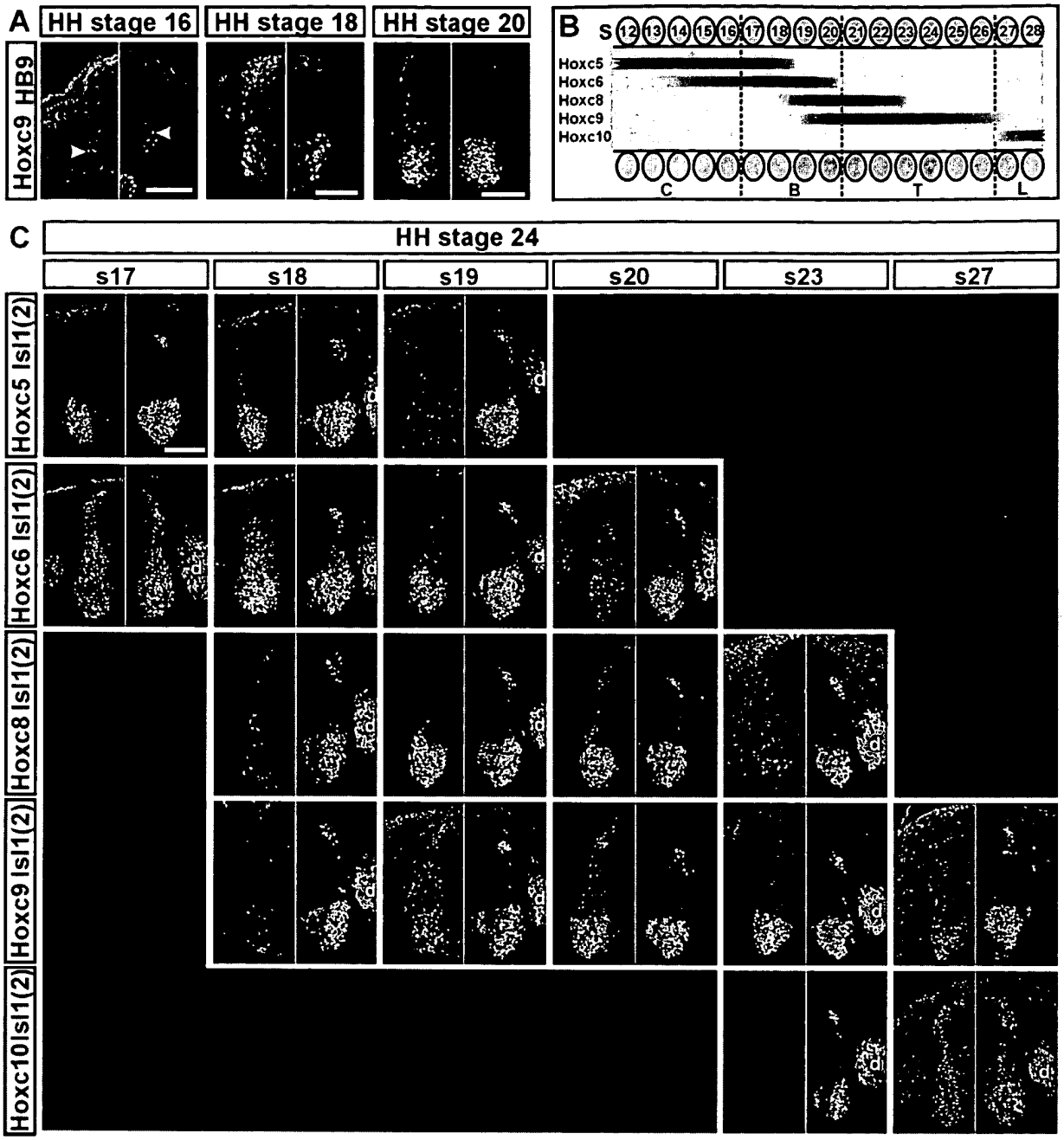

Figure 1. Hox-c Expression in Developing Spinal MNs

(A) Hoxc9 and HB9 expression in the spinal cord of HH stage 16–20 embryos. Arrowheads indicate Hoxc9$^+$ cells. Left side shows expression of Hoxc9, and right side Hoxc9 (red) and HB9 (green).

(B) R-C domains of Hoxc5 to Hoxc10 in the spinal cord of HH stage 24 embryos. Numbers in circles indicate somite level. C = cervical, B = brachial, T = thoracic, and L = lumbar levels.

(C) Expression of Hoxc5 to Hoxc10 at different levels of HH stage 24 embryos. Left side shows individual Hox-c proteins, and right side Hox-c (red) and Isl1(2) (green). "d" indicates dorsal root ganglion.

Scale bar: 100 μm.

rostral lumbar levels, and MNs generated in these explants expressed Hoxc9 and Hoxc10, but not Hoxc6 or Hoxc8 (Figures 3E and 3F). No expression of Hoxc5 was detected in these explants (Figures 3D, 7A, and data not shown). Thus, with the exception of Hoxc5, the R-C profile of Hox-c expression in MNs and other cell types appears to be specified soon after neural plate formation.

Induction of Neural Hox-c Expression by Hensen's Node

The early specification of neural Hox-c pattern led us to examine the origin of signals that establish this aspect of MN positional identity. Previous studies have provided evidence that the pattern of Hox expression is modifiable around the time of neural tube closure (Ensini et al., 1998; Lance-Jones et al., 2001). We reasoned that

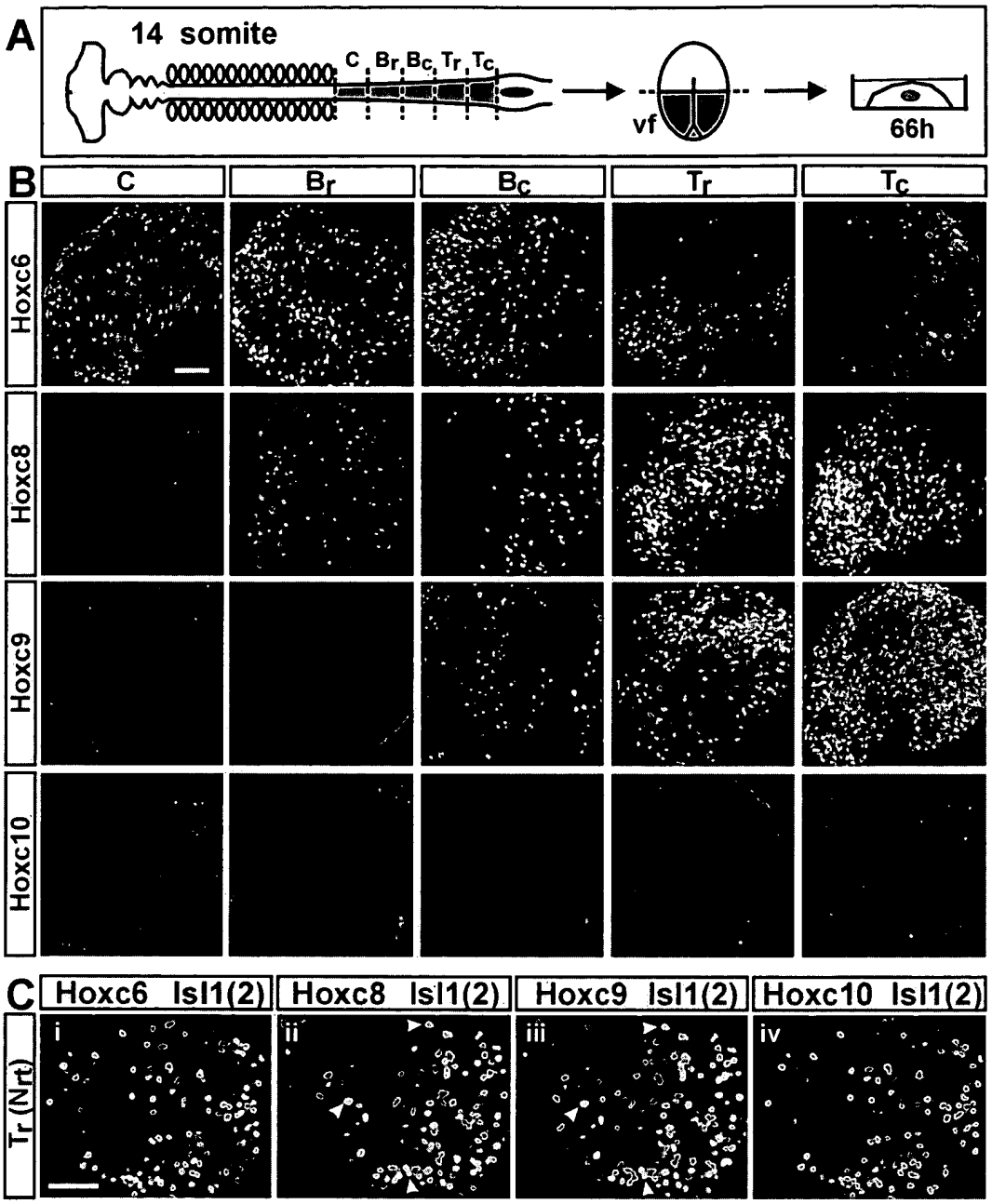

Figure 2. Expression of Hox-c in Neural Explants In Vitro

(A) Cells in neural tissue caudal to the somites and rostral to HN in 14s embryos populate cervical to thoracic regions of the spinal cord. (C = cervical, B_r= rostral brachial, B_c= caudal brachial, T_r = rostral thoracic, T_c = caudal thoracic). Ventral neural tissue including the floor plate (vf) was cultured for 66 hr.

(B) Expression of Hoxc6 to Hoxc10 in explants from (A).

(C) A T_r (N_{rt}) explant generates Hoxc$^+$, Isl1(2)$^+$ cells. Panels i and iv are the same section labeled with Hoxc6, Hoxc10, and Isl1(2). Panels ii and iii are the same section labeled with Hoxc8, Hoxc9, and Isl1(2). Arrowheads indicate Hoxc8$^+$, Hoxc9$^+$ MNs.

Scale bar: 50 μm.

neural plate tissue fated to give rise to rostral cervical levels, and lacking expression of Hoxc5 to Hoxc10 in vitro (Figures 3A and 3B), might still be competent to respond to extrinsic signals that impose a more caudal Hox-c identity. We therefore used ventral neural explants isolated from the rostral cervical level of 5s to 6s embryos (termed N_{rc} explants) (Figures 3A and 3B) in assays to define the source of Hox-c patterning activity. We also assayed the coexpression of Hox-c and Isl1(2) proteins to test whether the Hox-c expression pattern reflects MN positional identity and, in addition, determined the total number of Isl1(2)$^+$ MNs (Table 1).

From HH stage 6 onward, prospective spinal cord cells lie close to the rostral primitive streak and HN

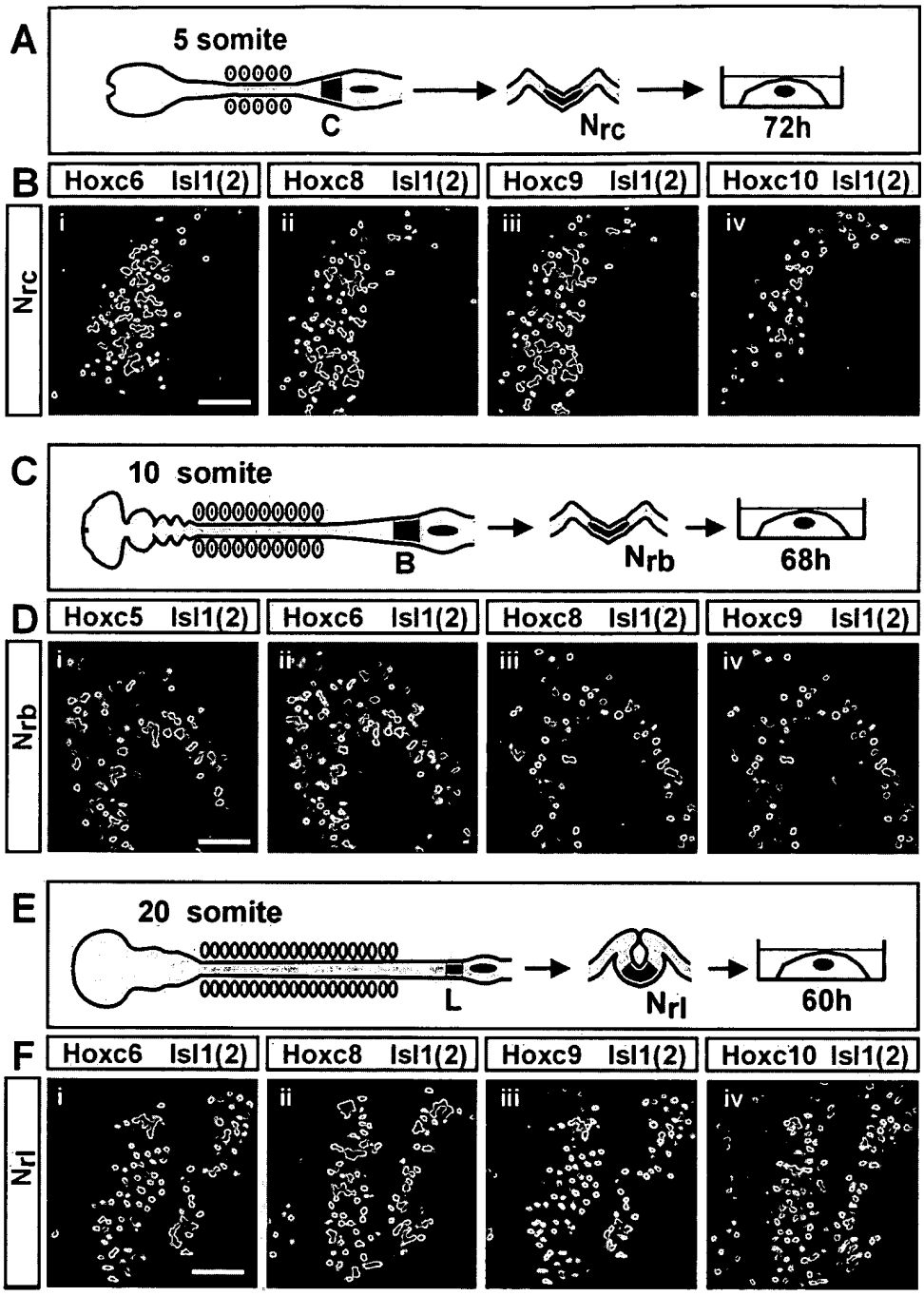

Figure 3. Hoxc6 to Hoxc10 Expression Is Specified at Neural Plate Stages

(A) Neural tissue located rostral to HN in 5s embryos populates rostral cervical levels of the spinal cord. Ventral neural plate (N_{rc}) tissue was cultured for 72 hr.

(B) N_{rc} explants do not express Hoxc6 to Hoxc10. Panels ii and iii are the same section labeled with Hoxc8, Hoxc9 and Isl1(2).

(C) Neural tissue located rostral to HN in 10s embryos populates rostral brachial levels. Ventral neural plate (N_{rb}) tissue was cultured for 68 hr.

(D) N_{rb} explants cultured in vitro express Hoxc6 and Hoxc8. Panels i and ii are the same section labeled with Hoxc5, Hoxc6, and Isl1(2). Panels iii and iv are the same section labeled with Hoxc8, Hoxc9, and Isl1(2).

(E) Neural tissue located rostral to HN in 20s embryos populates rostral lumbar levels. Ventral neural plate (N_{rl}) tissue taken from this region was cultured for 60 hr.

(F) N_{rl} explants express Hoxc9 and Hoxc10. Panels i and iii are the same section labeled with Hoxc6, Hoxc9 and Isl1(2). Panels ii and iv are the same section labeled with Hoxc8, Hoxc10, and Isl1(2).

Scale bar: 50 μm.

Table 1. Motor Neuron Number in Neural Explants Exposed to Different Factors

Explants + Reagents	Isl1 (2)$^+$ Cells/Section
N_{rc}	58 ± 11
N_{rc} + Fgf2 5 ng/ml	114 ± 15
N_{rc} + Fgf2 25 ng/ml	153 ± 11
N_{rc} + Fgf2 125 ng/ml	184 ± 16
N_{rc} + Fgf2 625 ng/ml	50 ± 4
N_{rc} + 5sHN	146 ± 15
N_{rc} + 15sHN	45 ± 7
N_{rc} + 15sHN + SU5402 12.5 M	46 ± 18
N_{rc} + Gdf8 10 ng/ml	33 ± 3
N_{rc} + Fgf2 25 ng/ml + Gdf8 10 ng/ml	143 ± 22
N_{rc} + Gdf8 40 ng/ml	43 ± 8
N_{rc} + Fgf2 25 ng/ml + Gdf8 40 ng/ml	102 ± 9
N_{rc} + RA 0.1 M	114 ± 22
N_{rt}	98 ± 8
N_{rt} + Fgf2 5 ng/ml	209 ± 44
N_{rt} + Gdf8 5 ng/ml	102 ± 15
N_{rt} + RA 0.1 M	154 ± 12

Isl1(2)$^+$ MNs were counted in sections of neural explants. Each value represents Isl1(2)$^+$ cells/section from 2 to 4 explants (mean ± SEM).

(Mathis et al., 2001; Schoenwolf, 1992), leading us to examine whether signals from these axial tissues are involved in the induction of neural Hox-c expression. Cells in N_{rc} explants grown in conjugate with HN tissue (see Experimental Procedures) were induced to express Hoxc6, Hoxc8, Hoxc9, and Hoxc10, but not Hoxc5 (Figure 4; data not shown). The precise profile of Hox-c expression depended on the developmental stage at which HN was isolated, and by inference the R-C position of HN. HN tissue derived from 5s to 6s embryos induced ~50 Hoxc6$^+$ cells (Hox-c$^+$ cell number/10 μm section), but no Hoxc8$^+$, Hoxc9$^+$, or Hoxc10$^+$ cells (Figure 4). HN tissue derived from 10s to 11s embryos induced ~80 Hoxc6$^+$ cells, 20 to 40 Hoxc8$^+$ and Hoxc9$^+$ cells, but no Hoxc10$^+$ cells (Figure 4). HN tissue derived from 14s to 15s embryos induced fewer (~30) Hoxc6$^+$ cells, slightly more (~30) Hoxc8$^+$ cells, many more (~120) Hoxc9$^+$ cells, and ~10 Hoxc10$^+$ cells (Figure 4). HN tissue from 19s to 20s embryos induced ~10 Hoxc6$^+$ cells, ~30 Hoxc8$^+$ cells, and additional Hoxc9$^+$ and Hoxc10$^+$ cells (Figure 4). In all instances, many induced Hox-c$^+$ cells were MNs, as assessed by coexpression of Isl1(2) (Table 1; data not shown). These findings provide evidence that signals from older and progressively more caudally positioned HN tissue induce a correspondingly more caudal profile of Hox-c expression.

We also examined whether tissues adjacent to HN induce neural Hox-c expression. N_{rc} explants were cultured together with caudal paraxial mesoderm, notochord, or ventral neural tissue isolated from 15s quail embryos. Newly formed notochord tissue at caudal thoracic level induced a few Hoxc6$^+$ cells, but did not induce Hox5$^+$, Hoxc8$^+$, Hoxc9$^+$, or Hoxc10$^+$ cells (data not shown). In contrast, older notochord tissue at rostral thoracic levels did not induce expression of Hox-c proteins (data not shown). Neither paraxial mesoderm nor ventral neural tube tissue induced expression of Hox-c proteins (data not shown). Thus, Hox-c-inducing activity is concentrated in HN, but is also expressed transiently in the notochord, a derivative of HN.

Concentration-Dependent Induction of Hox-c Expression by FGFs

Several fibroblast growth factor (FGF) genes, including *Fgf2*, *Fgf3*, *Fgf4*, and *Fgf8*, are expressed in HN and in the adjacent primitive streak (Crossley and Martin, 1995; Mahmood et al., 1995a; Niswander and Martin, 1992; Riese et al., 1995), leading us to analyze the Hox-c-inducing activity of FGFs. *Fgf8* is expressed in HN, primitive streak, and the tail bud over the time that the spinal Hox-c expression pattern is established, and the level of expression of *Fgf8* in HN and tail bud appeared to increase from the 6s to16s stages (Supplemental Figure S2 at http://www.neuron.org/cgi/content/full/32/6/997/DC1). In 16s stage embryos, *Fgf8* expression was also detected in caudal regions of the notochord (Supplemental Figure S2). We therefore tested whether FGF8, or other FGFs with similar activity, mimic the ability of HN to induce Hox-c expression in N_{rc} explants.

FGF8 activity requires heparin as a cofactor (Mahmood et al., 1995b; Storey et al., 1998; our observations). We therefore examined the Hox-c inductive ability of FGF8-adsorbed heparin beads, when grown in contact with N_{rc} explants for 72 hr. Heparin beads soaked in 11 μg/ml FGF8 induced expression of Hoxc6 in cells close to the bead, but did not induce Hoxc8, Hoxc9 ,or Hoxc10 (data not shown). Heparin beads soaked with 33 μg/ml FGF8 induced Hoxc6, Hoxc8, and Hoxc9 (data not shown), and heparin beads soaked with 100 μg/ml FGF8 induced Hoxc6 in distant cells, induced Hoxc8 and Hoxc9 in cells nearer to the beads, and induced Hoxc10 in cells adjacent to the beads (Supplemental Figure S3 on *Neuron* website). Hoxc5 was not detected in any of these conditions (data not shown). These results provide evidence that FGF8 can induce Hox-c expression in N_{rc} explants, and suggest that the profile of Hox-c expression varies with the concentration of FGF8.

To examine the influence of FGFs on Hox-c expression more quantitatively, we exposed N_{rc} explants to defined concentrations of FGF2, an FGF that mimics the activity of FGF8 in chick neural plate tissue (Muhr et al., 1999), and exhibits signaling activity in soluble form in the absence of heparin (Roghani et al., 1994). Exposure of N_{rc} explants to 5 ng/ml FGF2 induced ~20 Hoxc6$^+$, Isl1(2)$^+$ MNs (mean values/10 μm section), but no Hoxc8$^+$, Hoxc9$^+$, or Hoxc10$^+$ MNs (Figure 5A). Exposure of N_{rc} explants to 25 ng/ml FGF2 induced 17 Hoxc6$^+$, Isl1(2)$^+$ MNs; 25 Hoxc8$^+$, Isl1(2)$^+$ MNs; 36 Hoxc9$^+$, Isl1(2)$^+$ MNs; but no Hoxc10$^+$, Isl1(2)$^+$ MNs (Figure 5A). Exposure of N_{rc} explants to 125 ng/ml FGF2 induced 4 Hoxc6$^+$, Isl1(2)$^+$ MNs; 57 Hoxc8$^+$, Isl1(2)$^+$ MNs; 97 Hoxc9$^+$, Isl1(2)$^+$ MNs; and 5 Hoxc10$^+$, Isl1(2)$^+$ MNs (Figure 5A). Exposure of N_{rc} explants to 625 ng/ml FGF2 induced 1 Hoxc6$^+$, Isl1(2)$^+$ MN; 1 Hoxc8$^+$, Isl1(2)$^+$ MN; 20 Hoxc9$^+$, Isl1(2)$^+$ MNs; and 26 Hoxc10$^+$, Isl1(2)$^+$ MNs (Figure 5A). FGF2 failed to induce Hoxc5 at these concentrations (data not shown; Figure 7C). These results provide evidence that the FGF8/FGF2 class of FGFs mimic the ability of HN to induce patterned Hox-c expression, and indicate that FGFs act in a concentration-dependent manner that appears to reflect the age- and position-dependent signaling activity of HN.

To test if the induction of neural Hox-c expression by HN requires FGF signaling, we used SU5402, an effective inhibitor of FGF receptor 1 (FGFR1) activity (Moham-

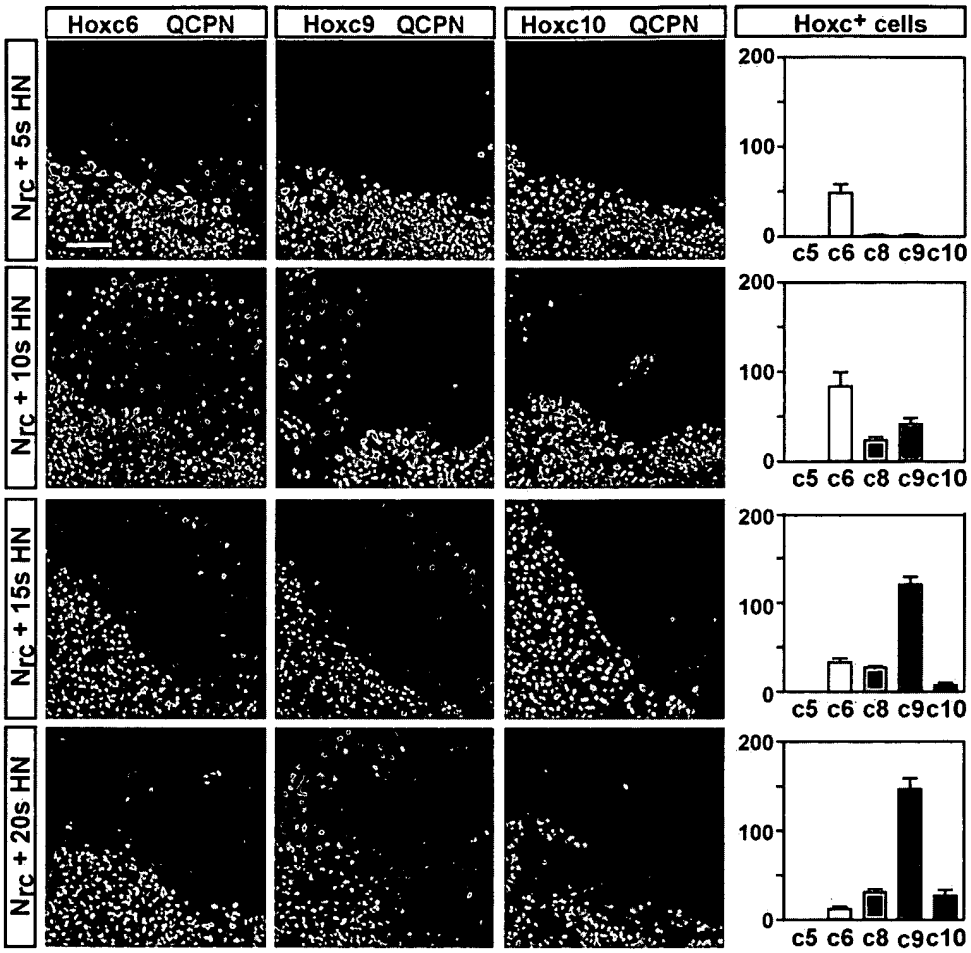

Figure 4. Induction of Neural Hox-c Expression by Hensen's Node

Coculture of HN tissue from 5s–20s quail embryos with N_{rc} explants induces the expression of Hoxc6 to Hoxc10 proteins, but not Hoxc5. Scale bar: 50 μm.

madi et al., 1997; Muhr et al., 1999; Streit et al., 2000; Wilson et al., 2000). Conjugates of chick N_{rc} explants and 15s quail HN tissue were grown in the presence of SU5402. The induction of neural Hoxc8, Hoxc9, and Hoxc10 expression by 15s quail HN was completely blocked by 5 μM SU5402 (data not shown). At this concentration, neural expression of Hoxc6 was not completely eliminated, but 12.5 μM SU5402 completely blocked expression of Hoxc6 as well as of Hox8, Hoxc9, and Hoxc10 (Figure 5B). The generation of Isl1(2)$^+$ MNs in these neural explants was not affected by SU5402 (Figure 5B, Table 1). These results provide evidence that the expression of Hoxc6 to Hoxc10 in MNs depends on FGF signals provided by HN.

An Accessory Role for Gdf11 in Patterning Neural Hox-c Expression

The high FGF concentration needed to induce a caudal profile of neural Hox-c expression prompted us to examine whether FGFs account completely for the Hox-c patterning activity of HN. Gdf11, a member of the transforming growth factor β (TGFβ) family is expressed in the tail bud region of mouse embryos (Gamer et al., 1999; Nakashima et al., 1999), and inactivation of *Gdf11* leads to caudal-to-rostral shift in the identity of paraxial mesodermal derivatives (McPherron et al., 1999). We therefore examined whether Gdf11 might contribute to the patterning of neural Hox-c expression. A chick *Gdf11* cDNA was isolated and its pattern of expression examined. *Gdf11* was not expressed in chick embryos prior to the 11s stage (Figure 6Ai), but from this stage, high levels of expression were detected in HN/tail bud and in caudal paraxial mesoderm (Figures 6Aii–6Aiv). This expression pattern persisted until HH stage 14 (data not shown).

We first examined whether Gdf11, like FGFs, can induce Hox-c expression in MNs in N_{rc} explants. Aggregates of HEK293 cells transfected with *Gdf11* were conjugated with N_{rc} explants, and Hox-c and Isl1(2) expression assayed after 72 hr. *Gdf11*-transfected HEK293 cells did not induce Hoxc6, Hoxc8, Hoxc9, or Hoxc10 expression in Isl1(2)$^+$ MNs (Figure 6C; data not shown). To determine more quantitatively the effect of this class of Gdf proteins on neural Hox-c expression, we also examined the activity of the closely related Gdf family member, Gdf8 (Gamer et al., 1999; Nakashima et al., 1999; Lee and McPherron, 2001). Exposure of N_{rc} explants to 10–40 ng/ml Gdf8

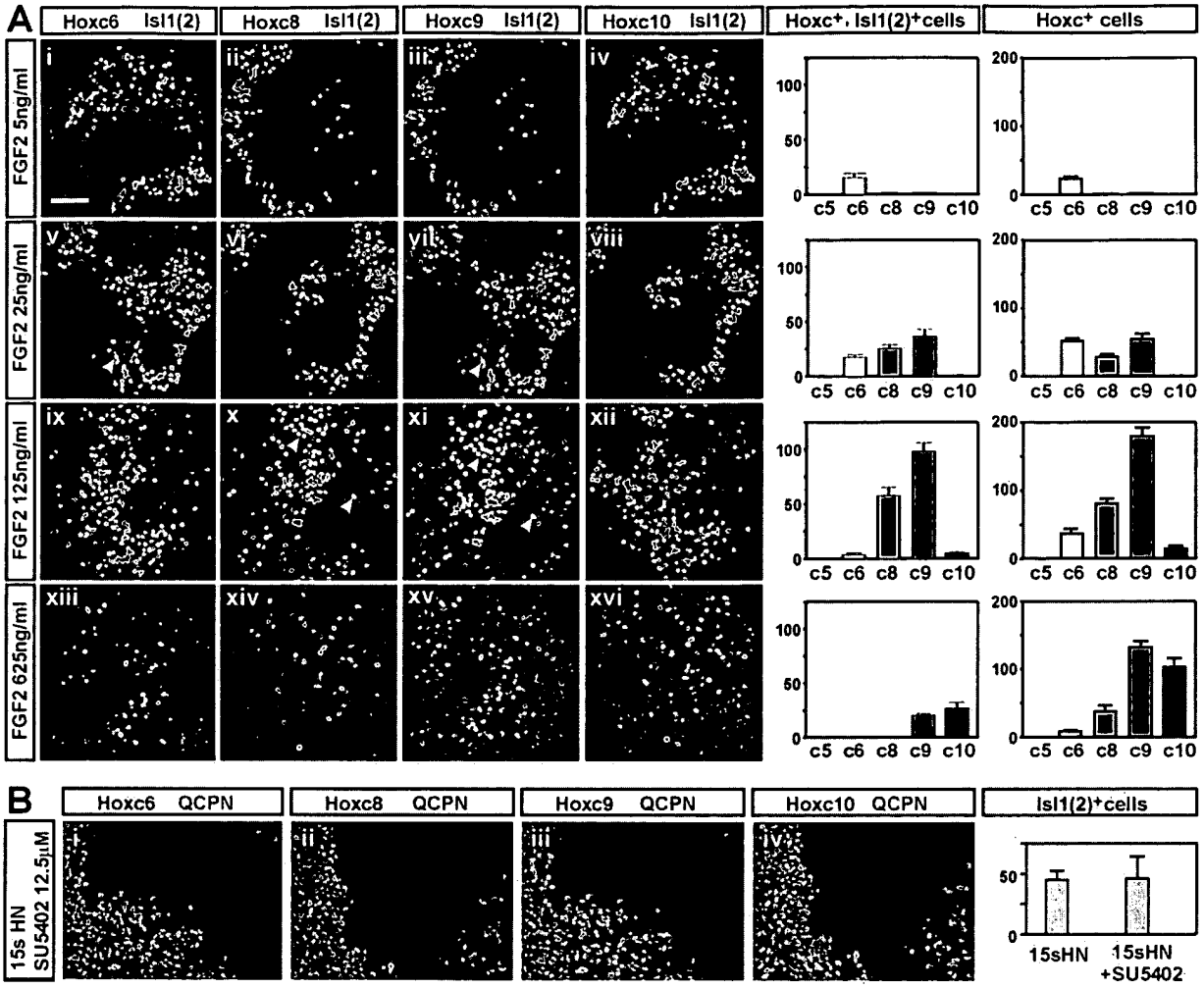

Figure 5. Concentration-Dependent Induction of Hox-c Expression by FGF2

(A) Induction of Hox-c expression by FGF2 in N_{rc} explants. Hox-c proteins are labeled in red and Isl1(2) in green. Panels i and iv, ii and iii, v and vii, vi and viii, x and xi, xiii and xv, and xiv and xvi are the same sections labeled with combinations of Hoxc and Isl1(2) antibodies. Arrowheads in panels v and vii indicate Hoxc6 and Hoxc9 double-labeled MNs, and arrowheads in panels x and xi indicate Hoxc8 and Hoxc9 double-labeled MNs. Hoxc5 expression was not induced.

(B) Addition of SU5402 (12.5 μM) blocks induction of Hox-c proteins in N_{rc} explants by 15s HN. The total number of Isl1(2)$^+$ cells was not affected.

Scale bar: 50 μm.

induced virtually no Hoxc6$^+$, Hoxc8$^+$, Hoxc9$^+$, or Hoxc10$^+$ MNs (Figures 6D and 6E). The number of MNs in N_{rc} explants was reduced in the presence of Gdf8 (Table 1), which may reflect an inhibitory influence on cell proliferation (Kalyani et al., 1998; Lee and McPherron, 2001). Exposure of cells to Gdf8 at these concentrations did not alter the D-V positional character of neural cells (data not shown). Thus, Gdf11 and Gdf8 appear to have little intrinsic Hox-c-inducing activity.

We next examined whether Gdf11/8 modulates the Hox-c patterning activity of FGFs. Exposure of N_{rc} explants to 25 ng/ml FGF2 alone induced Hoxc6$^+$, Hoxc8$^+$, and Hoxc9$^+$, but no Hoxc10$^+$ MNs (Figures 6B and 6I). N_{rc} explants conjugated with *Gdf11*-transfected HEK293 cells and exposed to 25 ng/ml FGF2 exhibited a 4-fold reduction in the number of Hoxc6$^+$ cells, and an ~2-fold increase in the number of Hoxc9$^+$ cells (Figure 6F). Similarly, when N_{rc} explants were exposed jointly to 10 ng/ml Gdf8 and 25 ng/ml FGF2, a 4-fold reduction in Hoxc6$^+$ MN number and a 3-fold increase in Hoxc9$^+$ MN number were observed (Figures 6G and 6J). Joint addition of 40 ng/ml Gdf8 and 25 ng/ml FGF2 virtually abolished expression of Hoxc6 in MNs, increased the number of Hoxc9$^+$ MNs 2-fold, and induced a few Hoxc10$^+$ MNs (Figures 6H and 6K). Together, these results provide evidence that Gdf11 enhances the ability of FGFs to induce a caudal Hox-c profile, thus promoting the differentiation of MNs with a more caudal positional identity.

A Role for Caudal Mesoderm in Refining Hox-c Expression

Thoracic level neural explants isolated from 14s embryos and grown in vitro aberrantly express Hoxc6 (Figure 2B), raising the possibility that tissues surrounding the neural tube normally have a role in limiting the poste-

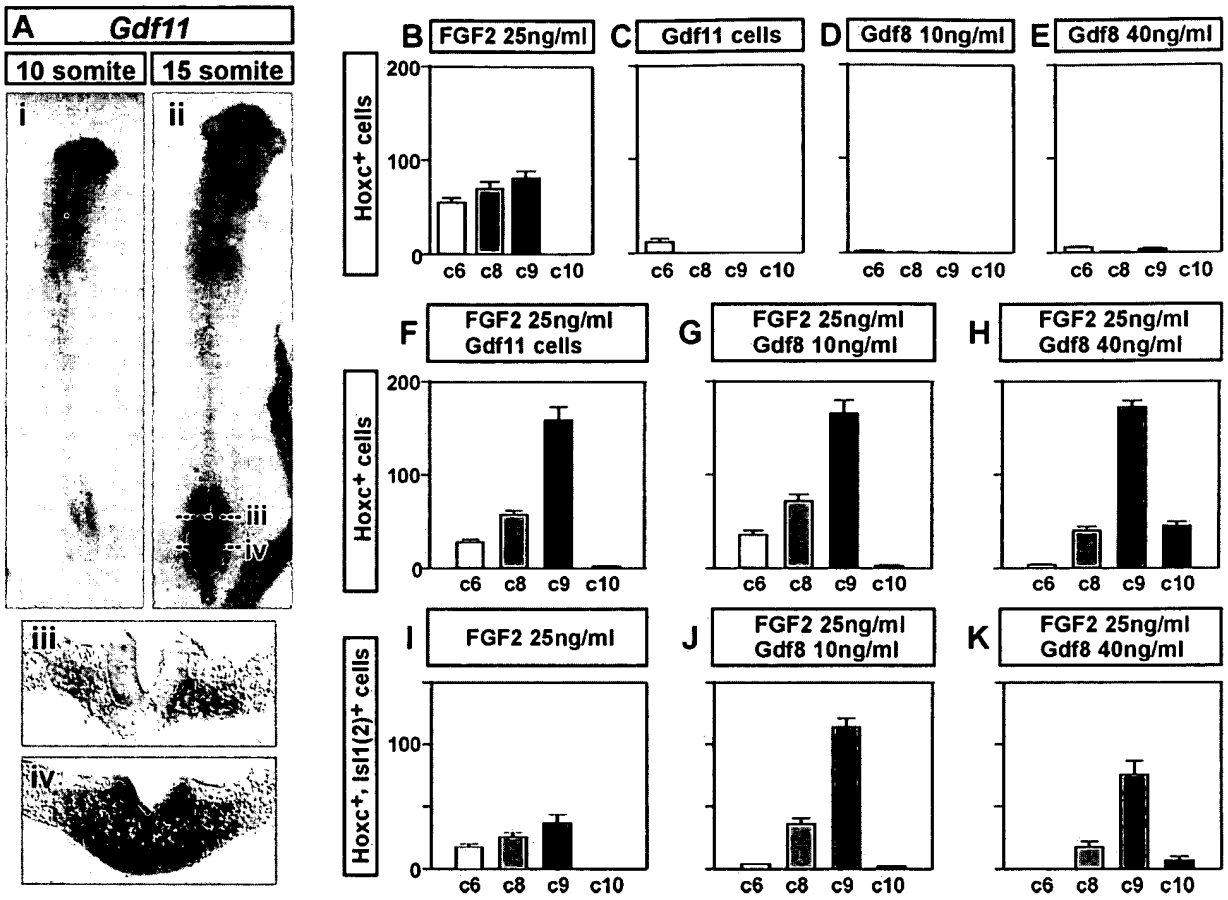

Figure 6. Regulation of Hox-c Expression by Gdfs

(A) Expression of *Gdf11* in chick embryos. (i) *Gdf11* is not detectable in embryos younger than 10s. (ii)–(iv) In older embryos, *Gdf11* is expressed by HN and caudal paraxial mesoderm. A low level of expression is detected in caudal neural plate. Dotted lines in (ii) indicate the levels of cross-sections shown in (iii) and (iv).

(B–K) Quantitation of Hox-c$^+$ cells in N_{rc} explants cultured in the presence of FGF2 and Gdfs.

rior extent of Hoxc6 expression. Since the paraxial mesoderm has been implicated in patterning the neural tube (Ensini et al., 1998; Grapin-Botton et al., 1997; Itasaki et al., 1996), we tested if signals from axial or paraxial mesoderm refine the caudal pattern of neural Hox-c expression. Rostral thoracic level neural tissue from 14s chick embryos (N_{rt} explants, Figures 2B and 2C) was conjugated with 14s quail caudal thoracic level paraxial mesoderm and/or notochord. N_{rt} explants conjugated with either paraxial mesoderm or notochord showed only a small reduction in the number of Hoxc6$^+$ cells, and a small increase in the number of Hoxc9$^+$ cells (data not shown). In contrast, N_{rt} explants conjugated with both paraxial mesoderm and notochord led to an almost complete loss of Hoxc6$^+$ cells, and to an ~2-fold increase in the number of Hoxc9$^+$ cells (Supplemental Figure S4 on *Neuron* website). These studies suggest that the combined actions of signals from thoracic level paraxial mesoderm and notochord repress Hoxc6 and enhance Hoxc9 expression in the thoracic neural tube.

Since *Fgf8* is expressed by posterior notochord and *Gdf11* by posterior paraxial mesoderm at levels flanking thoracic neural tube, we tested if these two factors account for the influence of mesodermal tissues on the pattern of Hox-c expression in the thoracic neural tube. Addition of FGF2 (5 ng/ml) or Gdf8 (5 ng/ml) alone to N_{rt} explants resulted in a small reduction in the number of Hoxc6$^+$ cells and a small increase in the number of Hoxc9$^+$ cells (data not shown). The joint addition of FGF2 (5 ng/ml) and Gdf8 (5 ng/ml) reduced the number of Hoxc6$^+$ cells by >70% and increased the number of Hoxc9$^+$ cells ~1.7-fold (Supplemental Figure S4 on *Neuron* website). Many of the resulting Hox-c$^+$ cells were MNs (data not shown). Thus, at thoracic levels, FGFs and Gdf11 expressed by notochord and posterior paraxial mesoderm, respectively, can act together to refine the profile of Hox-c expression established by signals from HN.

Retinoid Signaling from Rostral Paraxial Mesoderm Imposes an Anterior Profile of Neural Hox-c Expression

Signals from HN alone are not sufficient to impose a complete rostral profile of Hox-c expression since cervical level neural explants grown in the presence of either HN or FGFs fail to express Hoxc5. We therefore examined the source and nature of signals that confer the profile of Hox-c expression characteristic of the cervical level neural tube.

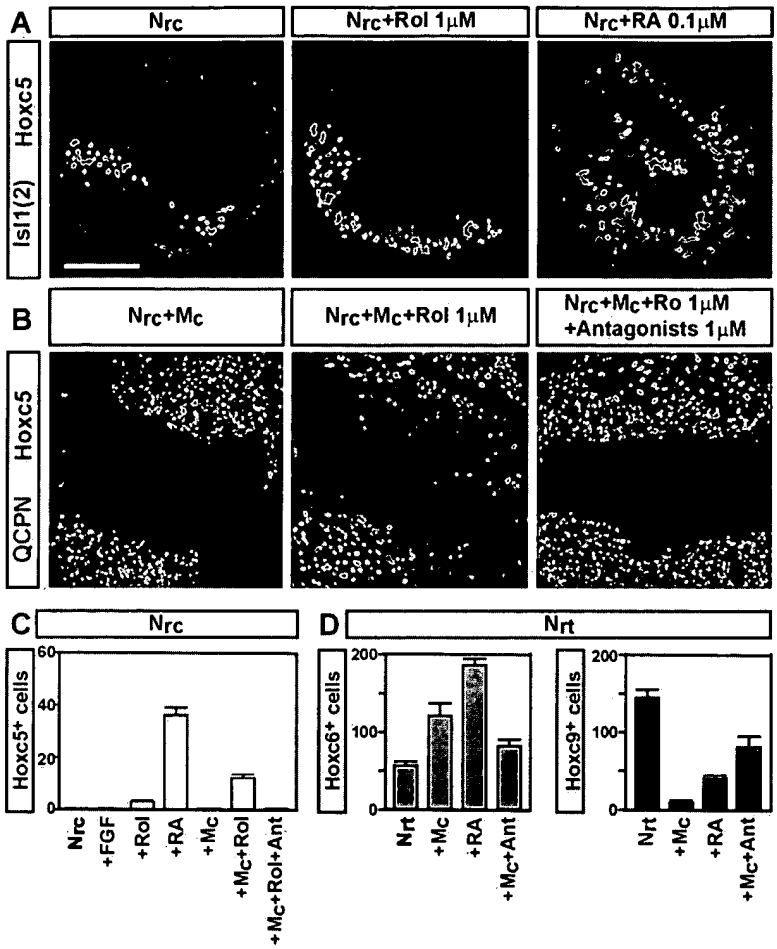

Figure 7. Regulation of Hox-c Expression by Cervical Level Paraxial Mesoderm and Retinoids

(A) N_{rc} explants cultured alone do not express Hoxc5. Addition of retinoic acid (0.1 μM) induces Hoxc5 expression. Many Hoxc5$^+$ cells are MNs, as revealed by Isl1(2) expression. Addition of retinol (1 μM) induces few Hoxc5$^+$ cells.

(B) Cervical level mesoderm (M_c) alone does not induce Hoxc5 in N_{rc} explants. In the presence of 1 μM retinol, induction of Hoxc5 is observed. The induction of Hoxc5 is blocked by RAR/RXR antagonists.

(C) Induction of Hoxc5$^+$ cells in N_{rc} explants by retinoids.

(D) Cervical level paraxial mesoderm and retinoids increase the number of Hoxc6$^+$ cells and decrease the number of Hoxc9$^+$ cells in N_{rt} explants.

Scale bar: 50 μm.

To address this issue, N_{rc} explants were grown together with mesodermal tissue isolated from quail embryos, or with candidate-inducing factors. Cervical level paraxial mesoderm expresses a high level of the retinoic acid (RA) synthesizing enzyme retinaldehyde dehydrogenase-2 (RALDH-2) (Berggren et al., 1999; Niederreither et al., 1997; Supplemental Figure S2B), and is a source of retinoid signals that influence the pattern of *Hox-b* expression in the hindbrain (Gould et al., 1998). We tested the involvement of retinoid signaling in the control of Hoxc5 expression by growing N_{rc} explants in the presence of cervical paraxial mesoderm and 1 μM retinol, a precursor of RA that permits efficient RA synthesis by paraxial mesoderm. N_{rc}-paraxial mesodermal conjugates grown for 72 hr contained many Hoxc5$^+$ neural cells, whereas cells in N_{rc} explants grown alone, or in 1 μM retinol, contained few if any Hox5$^+$ cells (Figures 7A–7C). N_{rc} explants grown in the presence of 0.1 μM RA without paraxial mesoderm or retinol also generated many Hoxc5$^+$ cells (Figures 7A and 7C). Of these Hoxc5$^+$ cells, ~40% were Isl1(2)$^+$ MNs (Figure 7A, data not shown). Exposure of 5s N_{rc} explants to 0.1 μM RA did not induce expression of Hoxc6, Hoxc8, Hoxc9, or Hoxc10 in MNs or other cells (data not shown), providing evidence that retinoids do not substitute for FGFs in the induction of these Hox-c proteins. Exposure of N_{rc} explants to retinoids produced a significant increase in total MN number (Table 1), consistent with the mitogenic effect of retinoids on neural progenitors (Sockanathan and Jessell, 1998). These results provide evidence that retinoids synthesized by the paraxial mesoderm induce expression of Hoxc5 in MNs. To test whether retinoid signals from paraxial mesoderm are required for the neural expression of Hoxc5, N_{rc} explants were conjugated with cervical level paraxial mesoderm in the presence of 1 μM retinol and the retinoic acid receptor (RAR) and retinoid X receptor (RXR) antagonists LG100815 (1 μM), and LG100849 (1 μM) (Sockanathan and Jessell, 1998). No neural expression of Hoxc5 was detected under these conditions (Figure 7B). These findings provide evidence that retinoid signals from cervical level paraxial mesoderm are required for the expression of Hoxc5 in MNs.

We next examined whether retinoid signaling from cervical level paraxial mesoderm also modifies the profile of Hoxc6, Hoxc8, and Hoxc9 expression—Hox-c proteins that are induced in MNs by FGF-mediated signals from HN. We exposed explants isolated from prospective thoracic levels of the neural tube (N_{rt} explants) to cervical level paraxial mesoderm or to retinoids. Conjugating 14s N_{rt} tissue with 14s cervical level paraxial mesoderm led to an ~2-fold increase in Hoxc6$^+$ cells and to a 3-fold decrease in Hoxc8$^+$ and a 6-fold decrease in Hoxc9$^+$ cells (Figure 7D, data not shown). 14s N_{rt} explants grown in the presence of 0.1 μM RA exhibited an ~2-fold increase in Hoxc6$^+$, Isl1(2)$^+$ MNs,

and an ~3-fold decrease in Hoxc8$^+$, Isl1(2)$^+$ and Hoxc9$^+$, Isl1(2)$^+$ MNs (Figure 7D, data not shown). The ability of cervical level paraxial mesoderm to increase Hoxc6 and decrease Hoxc8 and Hoxc9 expression was partially blocked by addition of RA receptor antagonists (Figure 7D, data not shown). Thus, retinoid signaling from cervical level paraxial mesoderm appears to refine the pattern of Hox-c expression induced in MNs by FGF signals from HN. These findings, together with the RA-mediated induction of Hoxc5, indicate that retinoid signaling from cervical level paraxial mesoderm is involved in conferring a complete rostral profile of Hox-c expression to MNs in the cervical spinal cord.

Patterning of Neural Hox-c Expression by FGF Signaling In Vivo

We next determined whether FGF signaling also regulates neural Hox-c expression in vivo. We misexpressed a mutated form of the FGF receptor 1 (FGFR1*) which activates FGF signal transduction in a ligand-independent manner (Hart et al., 2000). We reasoned that cells that express FGFR1* should behave as if they have been exposed to elevated levels of FGF signaling, and thus will undergo a rostral-to-caudal shift in their profile of Hox-c expression. We selected an activated FGF receptor 1 for these studies since the inhibitory activity of SU5402 on Hox-c expression implicates this FGF receptor subtype in neural patterning. FGFR1* was expressed unilaterally at brachial and cervical levels of the neural tube by electroporation into 9s–12s embryos, followed by analysis of neural Hox-c pattern at HH stages 20–21 and 24–25.

Expression of FGFR1* at brachial levels resulted in ectopic rostral expression of Hoxc9 and Hoxc10 in ventral regions of the spinal cord, coincident with local sites of ectopic FGFR1* expression (Figures 8C, 8D, 8G, and 8H). Some, but not all, ectopic Hoxc9$^+$ or Hoxc10$^+$ cells coexpressed MN markers. In contrast, FGFR1* expression suppressed the expression of Hoxc6 and to a lesser extent Hoxc8 from brachial level MNs (Figures 8E, 8E′, 8F, and 8F′). The ectopic rostral expression of Hoxc9 and Hoxc10 elicited by FGFR1* expression was also observed at more dorsal levels of the spinal cord (Figures 8C, 8D, 8G, and 8H). In some experiments, ectopic rostral expression of Hoxc9 and Hoxc10 could also be detected at cervical levels of the spinal cord (data not shown). Ectopic expression of an analogous wild-type FGFR1 had no effect on Hox-c pattern (Figures 8I–8L′; data not shown). Together, these results support the idea that FGF signaling regulates neural Hox-c pattern in vivo.

Discussion

Hox proteins control many features of vertebrate CNS development. In this study, we have examined the signals that establish the R-C pattern of Hox-c expression in MNs in the developing spinal cord. Our findings indicate that signals derived from HN initiate the R-C pattern of neuronal Hox-c expression, but the final pattern of Hox-c expression appears to depend on additional signals provided by the paraxial mesoderm (Figure 9). HN signals appear to be mediated in large part by FGFs, and the graded signaling activity of FGFs appears to specify many aspects of the position-dependent profile of neuronal Hox-c expression (Figure 9B). However, the complete profile of neuronal Hox-c expression at cervical levels of the spinal cord requires retinoid signaling from cervical level paraxial mesoderm. The profile of Hox-c expression at caudal thoracic and rostral lumbar levels appears to be refined by the activity of a TGFβ family member, Gdf11, expressed selectively at caudal levels in HN and the adjacent paraxial mesoderm (Figure 9). We discuss these findings in the context of the contributions of patterning signals from axial and paraxial mesoderm in specifying the positional identity of MNs.

Hensen's Node Signaling and the Initiation of Hox-c Expression

Signals from prospective axial mesodermal tissues, HN and the primitive streak, appear to induce Hox-c expression in MNs in a position-appropriate manner. Thus, HN tissue from embryos of progressively older stages induces a profile of Hox-c expression characteristic of progressively more caudal levels of the spinal cord. These findings extend previous observations that HN has age-dependent activities in specifying the fate of cells at midbrain and hindbrain levels of the neural axis (Kintner and Dodd, 1991; Storey et al., 1992). With the exception of Hoxc5, Hox-c inductive activity is largely confined to HN and the newly formed notochord.

The profile of Hox-c inductive activity exhibited by HN coincides well with the expression pattern of *FGF* genes, notably *Fgf8*. FGFs act in vitro in a graded manner, with higher concentrations of FGFs inducing a progressively more caudal profile of neural Hox-c expression. Similarly, activation of FGF receptor signaling in vivo induces a rostral-to-caudal shift in the profile of Hox-c expression. We note that in these in vivo studies, not all ectopic Hoxc9$^+$ and Hoxc10$^+$ cells located in the ventral spinal cord expressed MN markers, which may indicate additional actions of high level FGF signaling on MN differentiation. Nevertheless, together these in vitro and in vivo findings indicate that graded FGF signals derived from HN are likely to initiate the neural pattern of Hox-c expression. Such graded signaling could be achieved by a stage-dependent increase in the level of FGF signaling from HN since the level of *Fgf8* expression in HN appears to increase in older embryos. Alternatively, since neural cells fated to give rise to progressively more caudal regions of the spinal cord are positioned close to HN for progressively longer periods, they may be exposed to the same level of FGF signaling as cells destined to populate more rostral regions of the spinal cord, but for a longer period. Recent studies have provided evidence that FGF signaling within HN promotes the proliferation of prospective neural cells, maintaining a progenitor cell population throughout the period of spinal cord elongation (Mathis et al., 2001). Thus, FGF signaling within HN may coordinate the proliferation and R-C specification of spinal progenitor cells.

The onset of expression of the Hox-c proteins by spinal MNs occurs after neurons have left the cell cycle, yet our findings indicate that patterned Hox-c expression is specified at the time of neural plate formation. How is the early specification of positional identity linked to the

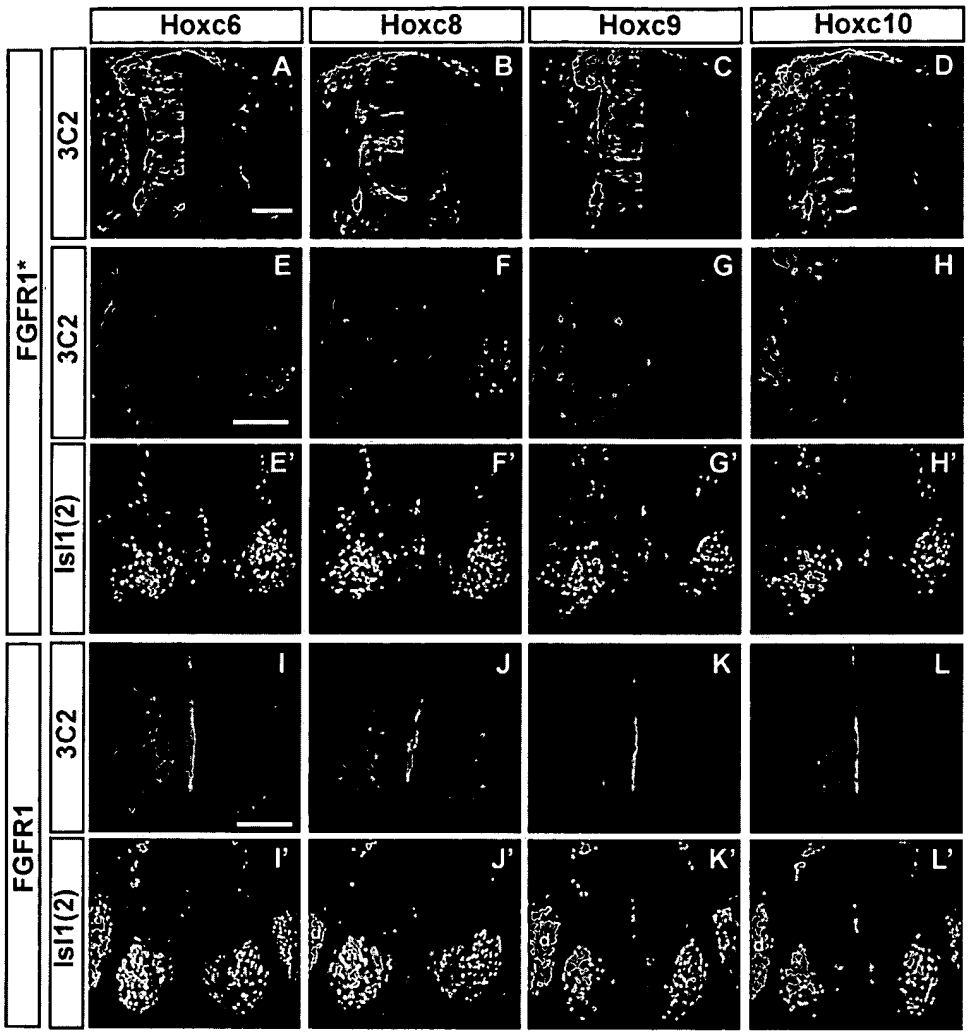

Figure 8. Repatterning of Hox-c Expression by Constitutively Activated FGFR1 In Vivo

(A–D) Patterns of ectopic Hoxc9 and Hoxc10 expression at brachial spinal cord 48 hr after electroporation of constitutively activated FGFR1 (FGFR1*) (HH stage 20–21).

(E–H and E'–H') 72 hr after electroporation of FGFR1* (HH stage 24–25), the induction of ectopic Hoxc9 and Hoxc10 and the repression of Hoxc6 and Hoxc8 are evident in domains of retroviral expression at brachial levels. Some but not all ectopic Hoxc9$^+$ and Hoxc10$^+$ cells are MNs, as assessed by Isl1(2) expression.

(I–L and I'–L') No repatterning of Hox-c expression is evident 72 hr after electroporation of wild-type FGFR1. "d" indicates dorsal root ganglia. Scale bars: 100 μm.

expression of Hox-c proteins in MNs? In *Xenopus*, the FGF-dependent regulation of *Hox* gene expression in mesodermal and neural cells involves *Cdx* genes (Isaacs et al., 1998; Pownall et al., 1996). Different members of *Cdx* gene family appear to be expressed at different R-C levels during early stages of chick neural development (Marom et al., 1997). Thus, *Cdx* genes are plausible mediators of FGF signaling in the regulation of Hox-c expression within MNs.

Signals from Paraxial Mesoderm Refine the Initial Pattern of Neural Hox-c Expression

Many aspects of the R-C pattern of Hox-c expression in spinal MNs can be accounted for by the action of FGFs provided by HN. But three observations indicate that additional signals are required to achieve the profile of Hox-c expression evident at cervical and lumbar levels. First, neither HN nor FGFs induce the neural expression of Hoxc5, a Hox-c protein that delineates cervical levels of the spinal cord. Second, segments of the thoracic neural tube isolated after the influence of HN-derived signals exhibit ectopic caudal expression of Hoxc6, suggesting that the normal caudal limit of Hoxc6 expression is defined by signals that act later than those provided by HN. Third, Hoxc10 expression is induced only at very high FGF concentrations, suggesting that the acquisition of a caudal Hox-c profile requires additional signals.

One source of these additional signals appears to be the paraxial mesoderm. Paraxial mesodermal signals refine the R-C pattern of neuronal Hox-c expression initiated by FGF signals from the primitive streak and HN. Rostral paraxial mesoderm expresses high levels

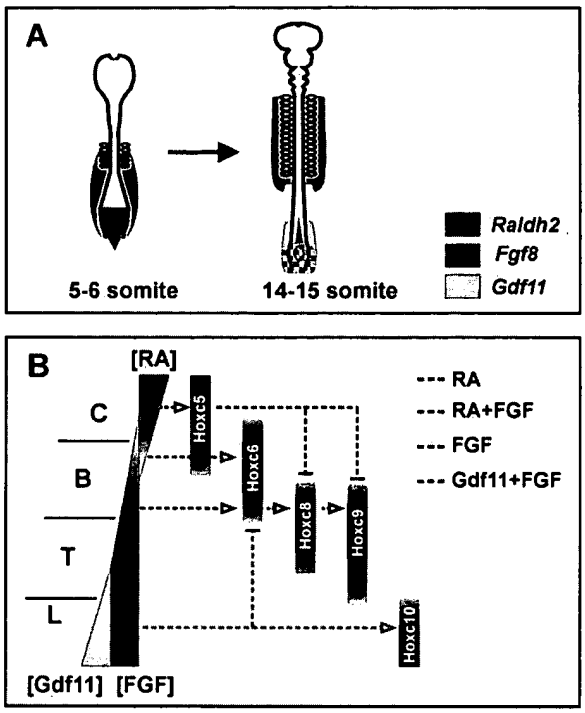

Figure 9. Convergent Signals Establish the Rostrocaudal Pattern of Hox-c Expression in Developing Spinal Cord

(A) Expression domains of *Raldh2*, *Fgf8*, and *Gdf11* in 5s–6s and 14s–15s embryos.

(B) Summary of combinatorial actions of FGFs, RA, and Gdf11 in specifying the R-C Hox-c profile in developing spinal cord. Low concentrations of FGF induce Hoxc6 and higher concentrations induce Hoxc8 and Hoxc9. In the cervical spinal cord, RA induces Hoxc5. In rostral brachial levels, RA also upregulates Hoxc6 and inhibits Hoxc8 and Hoxc9. At more caudal levels of the spinal cord, Gdf11 appears to act with FGFs to induce Hoxc10 and inhibit Hoxc6. The restricted distribution of these factors along the R-C axis of the embryo appears to establish the patterned expression of Hox-c proteins in MNs.

of retinoid signaling activity (Maden et al., 1998), and retinoids rather than FGFs induce the expression of Hoxc5 at cervical levels. Retinoid signaling also refines the expression pattern of Hox-c proteins whose expression is initiated by FGF signals from HN. Retinoid signaling from rostral paraxial mesoderm therefore appears necessary to establish a cervical profile of Hox-c expression in MNs.

Previous studies have implicated early retinoid signaling in establishing the generic character of the spinal cord (Muhr et al., 1999), and later retinoid signaling in defining the pattern of *Hox* gene expression in the developing hindbrain (Gavalas and Krumlauf, 2000; Niederreither et al., 2000). Both activities reveal that retinoids can exert a caudalizing influence on neural tissue. Our studies invoke a late role for retinoids in establishing regional identity within the spinal cord itself. Thus, paraxial mesodermal sources of retinoids appear to act at sequential developmental stages to impose different R-C positional values to cells in the spinal cord and hindbrain. In addition, in the context of spinal cord development, they indicate that retinoids impose a rostral rather than caudal positional character, and thus, inferences about rostralizing or caudalizing influences of retinoids depend on the specific domain of the neural axis under study.

Do retinoids have an equivalent role in R-C patterning at more caudal levels of the spinal cord? Paraxial mesoderm at prospective thoracic levels expresses *Raldh2*, but its onset of expression occurs at a later stage and its expression level is lower than that in the cervical level mesoderm (Berggren et al., 1999; Niederreither et al., 1997). By the time of its caudal expression, the initial specification of neural Hox-c expression has been established, and there has been a marked decrease in the competence of thoracic neural tissue to respond to retinoid signaling with changes in Hox-c expression (unpublished observations). At these more caudal levels, HN and the paraxial mesoderm selectively expresses Gdf11, a member of the TGFβ family. Gdf11 alone appears to have little Hox-c-inducing ability, but in conjunction with FGF signaling, markedly alters the profile of Hox-c expression. The prominent expression of Hoxc9 and Hoxc10 normally observed at caudal thoracic and rostral lumbar levels of the spinal cord may therefore be achieved through the joint exposure of neural cells to FGFs and Gdf11. A role for Gdf11 in establishing the lumbar character of the paraxial mesoderm has emerged from studies of *Gdf11* mouse mutants (McPherron et al., 1999). Our studies reveal a role for Gdf11 in patterning the neural tube, independent of its actions on mesodermal tissues, and raise the possibility that the actions of Gdf11 on caudal paraxial mesoderm may also reflect the modulation of FGF signaling.

The involvement of HN signaling in establishing the spinal Hox-c expression profile may help to explain the results of in vivo grafting studies that examined the source of signals that define the pattern of *Hoxd10* expression at the lumbar spinal cord. Here, signals from paraxial mesoderm are not sufficient to explain the neural pattern of *Hoxd10* expression (Lance-Jones et al., 2001). Our studies suggest that FGF and Gdf11 signals from HN may also control *Hox-d* expression at lumbar levels, in a manner similar to their role in patterning *Hox-c* expression.

Mesodermal Signaling and the Specification of MN Subtype Identity

Our studies also provide additional insights into the contributions of mesodermal signals to the specification of MN subtype identity. Signals from HN and the notochord, notably Shh and BMP antagonists, have been implicated in the D-V patterning events that establish the generic identity of MNs (Jessell, 2000; Liem et al., 2000; McMahon et al., 1998). Neither Shh nor BMP antagonists, however, appear to have a role in spinal MN subtype specification (Briscoe and Ericson, 2001). Instead, FGFs—a class of signals expressed by HN and transiently by the notochord—appear to be involved in controlling the subtype identity of spinal MNs. These results are consistent with the view that the specification of neuronal identity along the D-V and R-C axes of the spinal cord is controlled through largely independent signaling pathways. FGFs, retinoids, and Gdfs appear to alter progenitor cell proliferation in addition to regulating the Hox-c expression profile (Mathis et al., 2001; Socka-

nathan and Jessell, 1998; this study). As a consequence, MN number is altered by manipulations that influence neural Hox-c expression. Nevertheless, the change in neuronal Hox-c status appears to be achieved independently of controls on MN number.

Our studies have emphasized the key role of signals from the primitive streak and HN in establishing the R-C pattern of Hox-c expression. Other aspects of the R-C identity of spinal MNs may, however, be controlled by signals from the paraxial mesoderm. Specification of the LMC columnar subdivision of MNs is controlled by signals from limb level paraxial mesoderm that operate soon after neural tube closure (Ensini et al., 1998). Neither the patterns of *Gdf11* nor retinoid expression at these developmental stages readily explain this limb level-restricted signaling activity of the paraxial mesoderm. It is likely, therefore, that the paraxial mesoderm provides an additional signal devoted to the allocation of MN columnar identity. Are other features of MN subtype identity also controlled by mesodermal signals? Aspects of the pool identity of MNs within the LMC, defined by the projection pattern of motor axons and by expression of *ETS* genes, can be altered by changing the positional relationship between limb levels of the neural tube and adjacent paraxial mesoderm (Lin et al., 1998; Matise and Lance-Jones, 1996). Since in these studies grafted regions of the neural tube are manipulated at early developmental stages, it is unclear whether the establishment of motor pool identity depends solely on signals from paraxial mesoderm, or can be influenced by earlier signals from HN. Nevertheless, certain motor pool characteristics, notably *ETS* gene expression, are dependent on signals encountered by motor axons as they enter the lateral plate mesoderm at limb levels (Lin et al., 1998). Thus, three major mesodermal cell types, axial mesoderm, paraxial mesoderm, and lateral plate mesoderm, regulate distinct features of the R-C identity of spinal MNs, presumably through the provision of diverse extrinsic signals.

Experimental Procedures

Generation of Anti-Hox-c Antibodies

cDNAs corresponding to the N-terminal region of Hox-c proteins were subcloned into pGex5X vectors. Fusion proteins were purified and injected into mice, rabbits, or guinea pigs.

Cloning of Chick *Gdf11*

First strand cDNA was prepared with total RNA isolated from the posterior region of HH stage 13–14 embryos (Hamburger and Hamilton, 1951). A ~730 bp cDNA corresponding to amino acids 132–383 (missing aa 210–217) of human *GDF11* was generated by PCR using GARGARGAYGARTAYCA and CRTTRAARTANAGCATRTT primers. This clone shows 99% sequence conservation with human and mouse GDF11. (R = A or G, Y = C or T, N = A or T or C or G.)

Neural Explant Culture

Neural explants were isolated from different regions and stages of chick embryos. Their fates were determined by DiI labeling (see Supplemental Data at http://www.neuron.org/cgi/content/full/32/6/997/DC1).

HN tissue (all three germ layers) including rostral primitive streak was isolated from 5s–6s and 10s–11s quail embryos. Tail bud tissues (all three germ layers) were isolated from 14s–15s and 19s–20s quail embryos. Cervical level mesoderm tissue was isolated from regions anterior to HN in 5s–6s quail embryos (for conjugation with N_{rc}), or from regions posterior to the somites in 14s–15s quail embryos (for conjugates with N_{rt}). Thoracic level mesoderm and notochord tissues were isolated from regions anterior to HN (tail bud) in 14s–15s quail embryos. Tissues were cultured in serum-free medium in a collagen gel matrix (Liu and Jessell, 1998).

In Ovo Electroporation

RIS plasmids encoding replication in competent retroviruses that contain constitutively active FGFR1 (FGFR1*, RIS174HA) and control wild-type FGFR1 (RIS172HA) genes were generated using myristylated, cytoplasmic forms of the FGFR1 gene (Hart et al., 2000) into which an HA epitope tag was inserted. DNA (5 μg/μl) was electroporated into the neural tube in ovo using five pulses at 30 V; 50 ms. Embryos were analyzed at 48 hr (HH stage 20–21) or 72 hr (HH stage 24–25). The AMV-3C2 viral gag antibody made by D. Boettiger was obtained from the Developmental Studies Hybridoma Bank.

Preparation of Secreted Factors

Heparin beads (Sigma) were soaked with 11–100 μg/ml FGF8b (R&D system) (Storey et al., 1998) and conjugated with neural explants. FGF2 (Life Technologies), SU5402 (Calbiochem), Gdf8 (provided by S-J Lee), all-trans retinoic acid (Sigma), all-trans retinol (Sigma), LG100815, and LG100849 (Ligand Pharmaceuticals, provided by S. Sockanathan) were added at concentrations indicated.

pCI-neo (Promega) plasmids containing human *GDF11* and IRES-eGFP, or *BMP2* pro-region with *GDF11* mature region and IRES-eGFP, were transfected into HEK293 cells, selected with 1.5 mg/ml G418, and FACS sorted for GFP expression. Sorted cells were then transfected with an SPC1 expression plasmid (Constam and Robertson, 1999) to enhance processing to the mature GDF11 protein. After 24 hr, aggregates of 500–1000 cells were prepared in 20 μl hanging drops and conjugated with neural explants.

Immmunohistochemistry and Cell Counting

Explant tissues in collagen matrix were fixed and prepared for cryosection (Liu and Jessell, 1998). Details of antisera and immunohistochemical procedures are available as Supplemental Data on *Neuron* website.

Acknowledgments

We are grateful to S. Morton for help in generating antibodies, I. Schieren for FACS expertise, K. MacArthur for help in preparing the manuscript, I. Messina for technical assistance, M. Webster and D. Donoghue for the FGFR1 constructs, C. Tabin for *Hox-c* cDNAs, P. Gruss for *Hoxc8* and *Hoxc9* cDNAs, S.-J. Lee for *GDF11* cDNA and Gdf8 protein, A. Streit for *Fgf8* cDNA, and E. Robertson for the *SPC1* plasmid. We thank M. Ensini, B. Han, S. Sockanathan, A. Streit, and T. Tsuchida for advice; and S. Sockanathan, J. Dasen, and S. Zeitlin for comments on the manuscript. J.-P. L. is a recipient of a Burroughs Wellcome Fund Career Award in the Biomedical Sciences. E.L. was supported by Basil O'Connor Scholar Grant #5-FY99-855 from the March of Dimes Birth Defects Foundation and the HHMI Research Resources Program for Medical Schools. T.M.J. was supported by grants from NINDS and Project ALS, and is an HHMI Investigator.

Received August 14, 2001; revised November 15, 2001.

References

Bell, E., Wingate, R.J., and Lumsden, A. (1999). Homeotic transformation of rhombomere identity after localized Hoxb1 misexpression. Science *284*, 2168–2171.

Belting, H.G., Shashikant, C.S., and Ruddle, F.H. (1998). Multiple phases of expression and regulation of mouse Hoxc8 during early embryogenesis. J. Exp. Zool. *282*, 196–222.

Berggren, K., McCaffery, P., Drager, U., and Forehand, C.J. (1999). Differential distribution of retinoic acid synthesis in the chicken embryo as determined by immunolocalization of the retinoic acid synthetic enzyme, RALDH-2. Dev. Biol. *210*, 288–304.

Briscoe, J., and Ericson, J. (2001). Specification of neuronal fates in the ventral neural tube. Curr. Opin. Neurobiol. *11*, 43–49.

Carpenter, E.M., Goddard, J.M., Davis, A.P., Nguyen, T.P., and Capecchi, M.R. (1997). Targeted disruption of Hoxd-10 affects mouse hindlimb development. Development *124*, 4505–4514.

Constam, D.B., and Robertson, E.J. (1999). Regulation of bone morphogenetic protein activity by pro domains and proprotein convertases. J. Cell Biol. *144*, 139–149.

Crossley, P.H., and Martin, G.R. (1995). The mouse Fgf8 gene encodes a family of polypeptides and is expressed in regions that direct outgrowth and patterning in the developing embryo. Development *121*, 439–451.

de la Cruz, C.C., Der-Avakian, A., Spyropoulos, D.D., Tieu, D.D., and Carpenter, E.M. (1999). Targeted disruption of Hoxd9 and Hoxd10 alters locomotor behavior, vertebral identity, and peripheral nervous system development. Dev. Biol. *216*, 595–610.

Ensini, M., Tsuchida, T.N., Belting, H.G., and Jessell, T.M. (1998). The control of R-C pattern in the developing spinal cord: specification of MN subtype identity is initiated by signals from paraxial mesoderm. Development *125*, 969–982.

Ericson, J., Morton, S., Kawakami, A., Roelink, H., and Jessell, T.M. (1996). Two critical periods of Sonic Hedgehog signaling required for the specification of MN identity. Cell *87*, 661–673.

Forehand, C.J., Ezerman, E.B., Rubin, E., and Glover, J.C. (1994). Segmental patterning of rat and chicken sympathetic preganglionic neurons: correlation between soma position and axon projection pathway. J. Neurosci. *14*, 231–241.

Gamer, L.W., Wolfman, N.M., Celeste, A.J., Hattersley, G., Hewick, R., and Rosen, V. (1999). A novel BMP expressed in developing mouse limb, spinal cord, and tail bud is a potent mesoderm inducer in Xenopus embryos. Dev. Biol. *208*, 222–232.

Gavalas, A., and Krumlauf, R. (2000). Retinoid signalling and hindbrain patterning. Curr. Opin. Genet. Dev. *10*, 380–386.

Gavalas, A., Davenne, M., Lumsden, A., Chambon, P., and Rijli, F.M. (1997). Role of Hoxa-2 in axon pathfinding and rostral hindbrain patterning. Development *124*, 3693–3702.

Gould, A., Itasaki, N., and Krumlauf, R. (1998). Initiation of rhombomeric Hoxb4 expression requires induction by somites and a retinoid pathway. Neuron *21*, 39–51.

Grapin-Botton, A., Bonnin, M.A., and Le Douarin, N.M. (1997). Hox gene induction in the neural tube depends on three parameters: competence, signal supply and paralogue group. Development *124*, 849–859.

Hamburger, V., and Hamilton, H.L. (1951). A series of normal stages in the development of the chick embryo. J. Morphol. *88*, 49–92.

Hart, K.C., Robertson, S.C., Kanemitsu, M.Y., Meyer, A.N., Tynan, J.A., and Donoghue, D.J. (2000). Transformation and Stat activation by derivatives of FGFR1, FGFR3, and FGFR4. Oncogene *19*, 3309–3320.

Hollyday, M. (1980a). Organization of motor pools in the chick lumbar lateral motor column. J. Comp. Neurol. *194*, 143–170.

Hollyday, M. (1980b). Motoneuron histogenesis and the development of limb innervation. Curr. Top. Dev. Biol. *15*, 181–215.

Hollyday, M., and Hamburger, V. (1977). An autoradiographic study of the formation of the lateral motor column in the chick embryo. Brain Res. *132*, 197–208.

Isaacs, H.V., Pownall, M.E., and Slack, J.M. (1998). Regulation of Hox gene expression and posterior development by the Xenopus caudal homologue Xcad3. EMBO J. *17*, 3413–3427.

Itasaki, N., Sharpe, J., Morrison, A., and Krumlauf, R. (1996). Reprogramming Hox expression in the vertebrate hindbrain: influence of paraxial mesoderm and rhombomere transposition. Neuron *16*, 487–500.

Jessell, T.M. (2000). Neuronal specification in the spinal cord: inductive signals and transcriptional codes. Nat. Rev. Genet. *1*, 20–29.

Jungbluth, S., Bell, E., and Lumsden, A. (1999). Specification of distinct MN identities by the singular activities of individual Hox genes. Development *126*, 2751–2758.

Kalyani, A.J., Piper, D., Mujtaba, T., Lucero, M.T., and Rao, M.S. (1998). Spinal cord neuronal precursors generate multiple neuronal phenotypes in culture. J. Neurosci. *18*, 7856–7868.

Kania, A., Johnson, R.L., and Jessell, T.M. (2000). Coordinate roles for LIM homeobox genes in directing the D-V trajectory of motor axons in the vertebrate limb. Cell *102*, 161–173.

Kintner, C.R., and Dodd, J. (1991). Hensen's node induces neural tissue in Xenopus ectoderm. Implications for the action of the organizer in neural induction. Development *113*, 1495–1505.

Lance-Jones, C., Omelchenko, N., Bailis, A., Lynch, S., and Sharma, K. (2001). *Hoxd10* induction and regionalization in the developing lumbosacral spinal cord. Development *128*, 2252–2268.

Landmesser, L.T. (1978a). The development of motor projection patterns in the chick hind limb. J. Physiol. *284*, 391–414.

Landmesser, L.T. (1978b). The distribution of motoneurones supplying chick hind limb muscles. J. Physiol. *284*, 371–389.

Landmesser, L.T. (2001). The acquisition of motoneuron subtype identity and motor circuit formation. Int. J. Dev. Neurosci. *19*, 175–182.

Laskowski, M.B., and Sanes, J.R. (1987). Topographic mapping of motor pools onto skeletal muscles. J. Neurosci. *7*, 252–260.

Lee, K.J., and Jessell, T.M. (1999). The specification of dorsal cell fates in the vertebrate central nervous system. Annu. Rev. Neurosci. *22*, 261–294.

Lee, S.J., and McPherron, A.C. (2001). Regulation of myostatin activity and muscle growth. Proc. Natl. Acad. Sci. USA *98*, 9306–9311.

Liem, K.F., Jr., Jessell, T.M., and Briscoe, J. (2000). Regulation of the neural patterning activity of sonic hedgehog by secreted BMP inhibitors expressed by notochord and somites. Development *127*, 4855–4866.

Lin, J.H., Saito, T., Anderson, D.J., Lance-Jones, C., Jessell, T.M., and Arber, S. (1998). Functionally related MN pool and muscle sensory afferent subtypes defined by coordinate ETS gene expression. Cell *95*, 393–407.

Liu, J.P., and Jessell, T.M. (1998). A role for rhoB in the delamination of neural crest cells from the dorsal neural tube. Development *125*, 5055–5067.

Lumsden, A., and Krumlauf, R. (1996). Patterning the vertebrate neuraxis. Science *274*, 1109–1115.

Maden, M., Sonneveld, E., van der Saag, P.T., and Gale, E. (1998). The distribution of endogenous retinoic acid in the chick embryo: implications for developmental mechanisms. Development *125*, 4133–4144.

Mahmood, R., Kiefer, P., Guthrie, S., Dickson, C., and Mason, I. (1995a). Multiple roles for FGF-3 during cranial neural development in the chicken. Development *121*, 1399–1410.

Mahmood, R., Bresnick, J., Hornbruch, A., Mahony, C., Morton, N., Colquhoun, K., Martin, P., Lumsden, A., Dickson, C., and Mason, I. (1995b). A role for FGF-8 in the initiation and maintenance of vertebrate limb bud outgrowth. Curr. Biol. *5*, 797–806.

Marom, K., Shapira, E., and Fainsod, A. (1997). The chicken caudal genes establish an anterior-posterior gradient by partially overlapping temporal and spatial patterns of expression. Mech. Dev. *64*, 41–52.

Mathis, L., Kulesa, P.M., and Fraser, S.E. (2001). FGF receptor signalling is required to maintain neural progenitors during Hensen's node progression. Nat. Cell Biol. *3*, 559–566.

Matise, M.P., and Lance-Jones, C. (1996). A critical period for the specification of motor pools in the chick lumbosacral spinal cord. Development *122*, 659–669.

McMahon, J.A., Takada, S., Zimmerman, L.B., Fan, C.M., Harland, R.M., and McMahon, A.P. (1998). Noggin-mediated antagonism of BMP signaling is required for growth and patterning of the neural tube and somite. Genes Dev. *12*, 1438–1452.

McPherron, A.C., Lawler, A.M., and Lee, S.J. (1999). Regulation of anterior/posterior patterning of the axial skeleton by growth/differentiation factor 11. Nat. Genet. *22*, 260–264.

Mohammadi, M., McMahon, G., Sun, L., Tang, C., Hirth, P., Yeh, B.K., Hubbard, S.R., and Schlessinger, J. (1997). Structures of the tyrosine kinase domain of fibroblast growth factor receptor in complex with inhibitors. Science *276*, 955–960.

Muhr, J., Graziano, E., Wilson, S., Jessell, T.M., and Edlund, T. (1999). Convergent inductive signals specify midbrain, hindbrain, and spinal cord identity in gastrula stage chick embryos. Neuron *23*, 689–702.

Nakashima, M., Toyono, T., Akamine, A., and Joyner, A. (1999). Expression of growth/differentiation factor *11*, a new member of the BMP/TGFbeta superfamily during mouse embryogenesis. Mech. Dev. *80*, 185–189.

Niederreither, K., McCaffery, P., Drager, U.C., Chambon, P., and Dolle, P. (1997). Restricted expression and retinoic acid-induced downregulation of the retinaldehyde dehydrogenase type 2 (RALDH-2) gene during mouse development. Mech. Dev. *62*, 67–78.

Niederreither, K., Vermot, J., Schuhbaur, B., Chambon, P., and Dolle, P. (2000). Retinoic acid synthesis and hindbrain patterning in the mouse embryo. Development *127*, 75–85.

Niswander, L., and Martin, G.R. (1992). Fgf-4 expression during gastrulation, myogenesis, limb and tooth development in the mouse. Development *114*, 755–768.

Patten, I., and Placzek, M. (2000). The role of Sonic hedgehog in neural tube patterning. Cell. Mol. Life Sci. *57*, 1695–1708.

Pownall, M.E., Tucker, A.S., Slack, J.M., and Isaacs, H.V. (1996). eFGF, Xcad3 and Hox genes form a molecular pathway that establishes the anteroposterior axis in Xenopus. Development *122*, 3881–3892.

Riese, J., Zeller, R., and Dono, R. (1995). Nucleo-cytoplasmic translocation and secretion of fibroblast growth factor-2 during avian gastrulation. Mech. Dev. *49*, 13–22.

Roghani, M., Mansukhani, A., Dell'Era, P., Bellosta, P., Basilico, C., Rifkin, D.B., and Moscatelli, D. (1994). Heparin increases the affinity of basic fibroblast growth factor for its receptor but is not required for binding. J. Biol. Chem. *269*, 3976–3984.

Schoenwolf, G.C. (1992). Morphological and mapping studies of the paranodal and postnodal levels of the neural plate during chick neurulation. Anat. Rec. *233*, 281–290.

Sharma, K., Sheng, H.Z., Lettieri, K., Li, H., Karavanov, A., Potter, S., Westphal, H., and Pfaff, S.L. (1998). LIM homeodomain factors Lhx3 and Lhx4 assign subtype identities for MNs. Cell *95*, 817–828.

Sockanathan, S., and Jessell, T.M. (1998). MN-derived retinoid signaling specifies the subtype identity of spinal MNs. Cell *94*, 503–514.

Storey, K.G., Crossley, J.M., De Robertis, E.M., Norris, W.E., and Stern, C.D. (1992). Neural induction and regionalisation in the chick embryo. Development *114*, 729–741.

Storey, K.G., Goriely, A., Sargent, C.M., Brown, J.M., Burns, H.D., Abud, H.M., and Heath, J.K. (1998). Early posterior neural tissue is induced by FGF in the chick embryo. Development *125*, 473–484.

Streit, A., Berliner, A.J., Papanayotou, C., Sirulnik, A., and Stern, C.D. (2000). Initiation of neural induction by FGF signalling before gastrulation. Nature *406*, 74–78.

Studer, M., Lumsden, A., Ariza-McNaughton, L., Bradley, A., and Krumlauf, R. (1996). Altered segmental identity and abnormal migration of MNs in mice lacking Hoxb-1. Nature *384*, 630–634.

Tanabe, Y., and Jessell, T.M. (1996). Diversity and pattern in the developing spinal cord. Science *274*, 1115–1123.

Tiret, L., Le Mouellic, H., Maury, M., and Brulet, P. (1998). Increased apoptosis of motoneurons and altered somatotopic maps in the brachial spinal cord of Hoxc-8-deficient mice. Development *125*, 279–291.

Tsuchida, T., Ensini, M., Morton, S.B., Baldassare, M., Edlund, T., Jessell, T.M., and Pfaff, S.L. (1994). Topographic organization of embryonic MNs defined by expression of LIM homeobox genes. Cell *79*, 957–970.

Wilson, S.I., Graziano, E., Harland, R., Jessell, T.M., and Edlund, T. (2000). An early requirement for FGF signalling in the acquisition of neural cell fate in the chick embryo. Curr. Biol. *10*, 421–429.

Cell, Vol. 69, 1067–1070, June 26, 1992, Copyright © 1992 by Cell Press

Receptors for the TGF-β Family

Minireview

Joan Massagué
Cell Biology and Genetics Program
Howard Hughes Medical Institute
Memorial Sloan–Kettering Cancer Center
New York, New York 10021

It is roughly a decade since transforming growth factor β (TGF-β) first captured the attention of biologists. During this time, a large family of polypeptides related to TGF-β has been found, whose members all have wide-ranging effects on cell proliferation, differentiation, and organization (Figure 1; reviewed in Roberts and Sporn, 1990). These remarkably versatile factors act through mechanisms that remain an enigma, a fact that will change now that some of their receptors have been cloned. Although the receptor system that is emerging is not simple, and understanding its operation in detail will take considerable effort, the new vistas afforded by recent findings are worthy of commentary.

Many Binding Proteins: Are They All Receptors?

Membrane receptor labeling assays in a variety of cells have visualized up to nine distinct proteins that bind TGF-β (Figure 2). With binding constants below the nanomolar range, all these proteins seem to be important intermediaries between cells and TGF-β. The questions are, which of these proteins are signaling receptors, and what is the function of the others?

A partial answer comes from a set of chemically induced lung epithelial cell mutants that are insensitive to TGF-β (Laiho et al., 1991). Many of these mutants, all isolated by being resistant to growth inhibition by TGF-β, have lost the ability to bind TGF-β through receptors I and II. These glycoproteins of 53 kd and 75 kd, respectively, are present in essentially all nontransformed cells. The TGF-β-binding proteoglycan betaglycan (also known as receptor III), which is also widely distributed, is not lost in any of these mutants. Mutants that fail to bind TGF-β via receptors I and II lack not only the growth inhibitory response but also the gene responses typically induced by TGF-β. In addition, receptors I and II are the only TGF-β-binding proteins expressed in some responsive cell types. These observations suggest that receptors I and II may mediate multiple TGF-β effects and that betaglycan might be dispensable for responsiveness to this factor.

TGF-β receptors I and II can coexist with other TGF-β-binding proteins besides betaglycan. Vascular endothelial cells that lack betaglycan express a disulfide-linked dimer of 95 kd subunits that binds TGF-β1 but not TGF-β2. In certain cells, three proteins that are attached to the cell membrane via phospholipid anchors also bind either TGF-β1 or β2. A large (~400 kd) cell surface TGF-β-binding protein has been isolated from bovine liver. A 64 kd protein seen only in rat pituitary cells is unique in that it binds TGF-β, activin, and inhibin. None of these TGF-β-binding proteins is known to function as a signaling receptor, which at the moment renders questionable the numerical receptor nomenclature (e.g., betaglycan as TGF-β receptor III).

The members of the TGF-β family may signal through families of related receptors. In fact, activin A and BMP-4 bind to membrane proteins that are similar in size to TGF-β receptors I and II. Direct evidence, however, comes from the recent cloning of type II receptors for activin and TGF-β, as well as receptors for yet unknown ligands. These receptors form a family of transmembrane protein serine/threonine kinases.

Type II Receptors: A Serine/Threonine Kinase Receptor Family

The first member of this family identified in animal cells is Caenorhabditis elegans *daf-1*, a gene required for formation of a dauer larva (which is resistant to environmental stress) in response to nutrient limitation. Although the Daf-1 ligand has not been identified, it might be encoded by *daf-8* or *daf-14*, two genes upstream of *daf-1* in the same pathway (Georgi et al., 1990). Other cloned members of this family include the human type II TGF-β receptor (TβR-II; Lin et al., 1992) and two mouse type II activin receptors, ActR-II (Mathews and Vale, 1991) and ActR-IIB (Attisano et al., 1992). ActR-II and TβR-II have been isolated by expression cloning in mammalian cells. ActR-IIB was identified on the basis of its homology to ActR-II and *daf-1*.

The ectodomain of these receptors is small—roughly half the size of the ligands—and rich in cysteine (Figure 3). The TGF-β, activin, and Daf-1 receptors show limited ectodomain sequence similarity (e.g., see Lin et al., 1992). This contrasts with the similarity between activins and TGF-βs, including conservation of their nine cysteines.

The cytoplasmic region comprises primarily the kinase domain, in which the highest level of similarity is observed

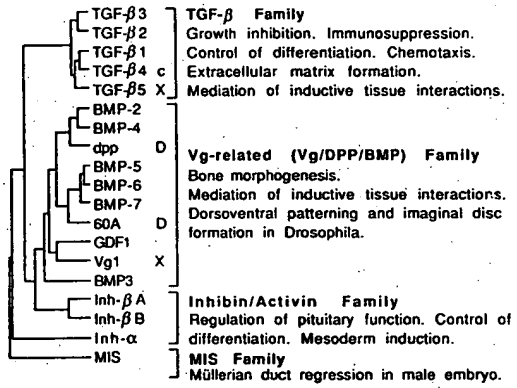

Figure 1. The TGF-β Superfamily and Some Representative Activities

The hypothetical relationship dendrogram (Geneworks, Intelligenetics) is based on the protein sequence of the bioactive domains of these factors. All factors are human except as indicated: c, chicken; X, Xenopus; and D, Drosophila. All factors act as dimers. Activins are dimers of βA and/or βB chains, and inhibins are dimers of one α chain and one βA or βB chain.

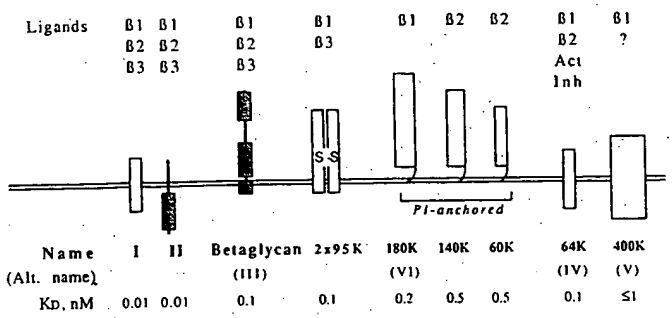

Figure 2. Membrane-Associated TGF-β-Binding Proteins

The cloned binding proteins are shaded. β1, β2, and β3, the three TGF-β isoforms; Act, activin A; Inh, inhibin A.

among family members. Its sequence fits better the serine/threonine kinase consensus than the tyrosine kinase consensus. Bacterially expressed TβR-II cytoplasmic region does show serine/threonine autophosphorylating activity (Lin et al., 1992). Neither ligand-induced activation of the kinase nor kinase activity toward exogenous substrates have yet been described. A distinguishing feature of the kinase domains of type II receptors is the presence of two short inserts. Mutation of a conserved proline in the second insert disrupts the function of *daf-1* (Georgi et al., 1990). The kinase domain is followed by a short C-terminal tail that contains potential phosphorylation sites.

A given ligand may interact with various receptor isoforms, as in the case of activin. The repertoire of activin receptors includes five protein isoforms derived from two genes (ActR-II and ActR-IIB). Four isoforms are generated by alternative splicing of short sequences flanking the transmembrane domain in ActR-IIB. Isoforms that retain the external juxtamembrane segment show the highest affinity for activin (Attisano et al., 1992). This receptor diversity amplifies the regulatory potential of the system.

Protein serine/threonine kinases are, therefore, likely to be used by TGF-β family members to signal their many actions. These receptors represent a novel class of transmembrane signaling molecules. Many cytoplasmic and nuclear serine/threonine kinases have been characterized, and tyrosine kinases are common among mitogen receptors; however, the type II receptors are novel in animal cells as transmembrane serine/threonine kinases. It will be important to identify the relevant substrates for these kinases and to confirm that the kinase activity is indeed toward serine and threonine and required for cell stimulation through these receptors. Further, it will be important to determine whether type II receptors can signal alone or as part of a signaling receptor complex.

A Signaling Receptor Complex?

By analogy with the receptor tyrosine kinases (Ullrich and Schlessinger, 1990), ligand-induced dimerization may suffice to activate the serine/threonine kinase receptors. Receptor dimerization ought to be facilitated by the fact that TGF-β and related factors are themselves dimers. With a functional binding site in the ectodomain and an enzymatic device in the cytoplasmic domain, the type II receptors might not need any other subunit to initiate a response. Indeed, injection of cDNAs encoding ActR-II or ActR-IIB increases the responsiveness of Xenopus embryo explants to activin (Kondo et al., 1991; Mathews et al., 1992). Introduction of receptor serine/threonine kinases into cells that lack other binding proteins might be sufficient to confer responsiveness to the corresponding factors.

Things may not be quite this simple, though. In at least some cell types, expression of receptor II alone may not be sufficient for TGF-β responsiveness. Some of the cell mutants resistant to TGF-β show normal binding of TGF-β to receptor II but have lost binding to receptor I (Laiho et al., 1991). These cells are as resistant to TGF-β action as receptor II-defective cells. Further, mutants defective only in receptor I belong to complementation group distinct from mutants defective in both receptors. Cell hybrids between the two rescue expression of functional receptor I and full responsiveness to TGF-β. This suggests that TGF-β binding to receptor II might be needed for binding

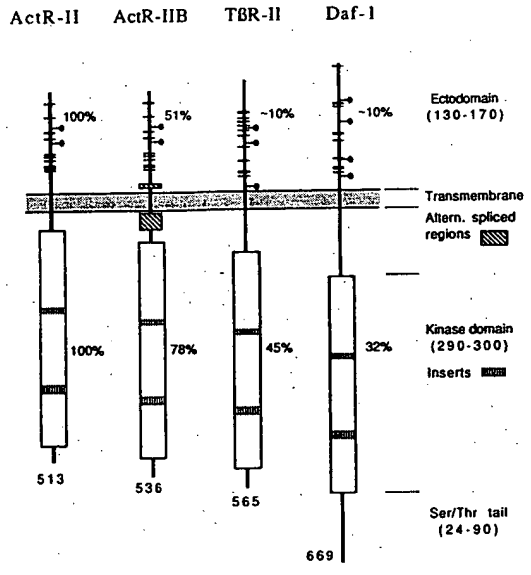

Figure 3. The Protein Serine/Threonine Kinase Receptor Family

The percent similarity between the ectodomain of each receptor and that of Act-RII as well as the similarity for the the kinase domain are indicated to the right of each domain. Numbers in parentheses, the number of amino acids in the domains; numbers below each receptor, the total number of amino acids; bars, cysteine residues; pins, potential N-glycosylation sites.

to receptor I, both receptor components then cooperating to initiate a productive signal. However, no physical association between receptors I and II has yet been reported, and this model has been challenged (Geiser et al., 1992) on the basis that some human carcinoma cell lines with low levels of receptor II show still receptor I and gene responses to TGF-β. This model can now be tested with the cloned TβR-II.

If receptor I proves to be important for TGF-β signaling, what might its function be? Several possibilities can be envisaged. Receptor I could be a modulator of ligand binding, a subunit needed to activate the kinase, or a modifier or amplifier of the kinase signal. Alternatively, it could constitute either a site for docking and presentation of substrates to the kinase or an independent signaling molecule. Further definition of this protein on a molecular level seems important.

The Conflict between Receptor Affinity and Biopotency of TGF-β Isoforms

There seems to be pressure to retain certain functional differences between TGF-β1, TGF-β2, and TGF-β3. However modest, the structural differences between these isoforms are strictly conserved throughout evolution (reviewed in Roberts and Sporn, 1990). Receptors I and II can indeed discriminate between them; overall, they bind TGF-β1 and TGF-β3 better than they bind TGF-β2. Yet, in many assays, all three TGF-β isoforms paradoxically show similar potency, which on the surface correlates well with the relative ability of these isoforms to bind to betaglycan.

Pointing to a possible resolution of this conflict, it has been observed that, whereas a large fraction of receptors I and II has high affinity for TGF-β1 and TGF-β3 but not for TGF-β2, a smaller fraction of receptors displays high affinity for all three TGF-βs (Cheifetz et al., 1990). What makes this receptor subset different from the rest is not known, but most cells in culture seem to express sufficient levels of it to ensure high sensitivity to all three TGF-βs. However, in certain vascular endothelial cells and hematopoietic progenitor cells, TGF-β2 can indeed be much weaker than TGF-β1 or β3 as a growth inhibitor. Interestingly, these two cell types have little or no betaglycan. Might betaglycan or some of the other TGF-β-binding proteins modulate the relative ligand isoform affinity of the signaling receptors?

Receptor Accessories: Betaglycan and Others

Betaglycan is the most widely distributed TGF-β-binding protein after receptors I and II (Wang et al., 1991; López-Casillas et al., 1991, and references therein). Betaglycan is a membrane-anchored proteoglycan that binds TGF-β via the core protein. In most cell lines, the glycosaminoglycan chains of betaglycan are a mix of heparan sulfate and chondroitin sulfate (Segarini and Seyedin, 1988). The glycosaminoglycan chains are dispensable for cell surface expression of betaglycan or for TGF-β binding to it. However, the heparan sulfate chains can bind fibroblast growth factor (Andres and Massagué, 1992). Thus, betaglycan might function as a receptor accessory molecule in both the TGF-β and FGF systems.

Betaglycan is an 853 aa protein with a remarkable mosaic structure (Wang et al., 1991; López-Casillas et al., 1991). The extracellular region has two cysteine-containing domains separated by a spacer region. The transmembrane and short cytoplasmic region are highly (63%) identical to the corresponding regions of endoglin, a disulfide-linked dimer of 95 kd subunits expressed in vascular endothelium that is of unknown function (Gougos and Letarte, 1990). A weaker similarity to endoglin is detected in the ectodomain of betaglycan, particularly within the first cysteine domain. The second cysteine domain of betaglycan shows similarity to various membrane-associated proteins of diverse function (Bork and Sander, 1992). These include the sperm receptors ZP2 and ZP3, uromodulin (a kidney-derived urinary protein), and the major pancreatic secretory granule membrane protein GP-2. The significance of this similarity is not known.

The absence of a discernable signaling device in the cytoplasmic domain of betaglycan as well as what is known about its biology do not suggest a direct role in TGF-β signaling. That its ectodomain is cleaved and released into the medium supports the notion that betaglycan might act as a pericellular regulator of TGF-β access to the cell (López-Casillas et al., 1991). Betaglycan might, for example, bind TGF-β surplus in the pericellular environment for presentation to the signaling receptors, thus acting as a reservoir or capacitor for this receptor system. Consistent with this view, betaglycan overexpression appears to enhance TGF-β1 binding to receptor II (Wang et al., 1991). It would still be premature, however, to discard the possibility of a more direct interaction between betaglycan and the signaling TGF-β receptors. These considerations also apply to the other TGF-β-binding proteins.

Links to Tumor Suppressor Genes

A fascinating aspect of the biology of TGF-βs is their ability to inhibit cell proliferation (Moses et al., 1990). TGF-β operates through a signaling system that can override the action of mitogens. This raises the question of whether disruption of this system leads to unconstrained cell proliferation and progression towards malignancy. That is, do the genes encoding the components of this system—the TGF-β receptors and signal translators—belong to the class known as tumor suppressor genes (Weinberg, 1991)? The converse question is also raised: do any of the known tumor suppressor gene products participate in TGF-β signaling? The answers could well be affirmative. Certain tumor cell lines lack TGF-β receptors, a condition not seen in nontransformed cells (Kimchi et al., 1988). There are still few known cases of complete TGF-β receptor loss, and no causal link has yet been proven between this anomaly and tumor progression. But this may change as more probes to detect receptor defects become available.

TGF-β is a part-time growth inhibitor. In some cases, TGF-β may even facilitate cell growth by inducing expression of autocrine mitogens, or by inducing production of extracellular matrices that support cell proliferation (Moses et al., 1990). Therefore, not every piece of its signaling system may be regarded as a potential tumor suppressor gene product. Some TGF-β receptor forms might be better equipped than others to signal growth inhibition. It is not clear, however, that growth inhibition is specified by TGF-β receptors that are distinct from those that specify other

responses. On the contrary, loss of growth inhibition by TGF-β can be accompanied by loss of other responses such as control of extracellular matrix production (Laiho et al., 1991). The circuitry that couples TGF-β receptor signals to negative regulators of cell cycle might be the main determinant of growth inhibitory responsiveness to this factor.

This circuitry may involve tumor suppressor gene products, such as the retinoblastoma susceptibility gene product, RB. RB is thought to act as an impediment to cell cycle progression through the late portion of G1, unless it becomes phosphorylated in middle G1. TGF-β prevents RB phosphorylation in cells whose growth is inhibited by TGF-β (Laiho et al., 1990). Other evidence implicates RB in the shutdown of the c-*myc* gene by TGF-β; in this case, cells are deprived of c-myc protein, which is required for the completion of G1 (Pietenpol et al., 1990). The mechanistic details of these responses are still unclear, but these findings illustrate how an antimitogen may engage the services of a tumor suppressor gene product in order to inhibit cell growth.

The progress reviewed here is helping to understand the process of cell stimulation by the TGF-β family and brings closer the possibility of manipulating this process for therapeutic purposes. Some of the interesting questions mentioned here will soon be addressed. As is often the case when enigmas are about to unravel, we may anticipate quite a few unexpected answers.

References

Andres, J. L., and Massagué, J. (1992). J. Biol. Chem. *267*, 5927–5930.

Attisano, L., Wrana, J. L., Cheifetz, S., and Massagué, J. (1992). Cell *68*, 97–108.

Bork, P., and Sander, C. (1992). FEBS Lett. *300*, 237–240.

Cheifetz, S., Hernandez, H., Laiho, M., ten Dijke, P., Iwata, K. K., and Massagué, J. (1990). J. Biol. Chem. *265*, 20533–20538.

Geiser, A. G., Burmester, J. K., Webbink, R., Roberts, A. B., and Sporn, M. B. (1992). J. Biol. Chem. *267*, 2588–2593.

Georgi, L. L., Albert, P. S., and Riddle, D. L. (1990). Cell *61*, 635–645.

Gougos, A., and Letarte, M. (1990). J. Biol. Chem. *265*, 8361–8364.

Kimchi, A., Wang, X.-F., Weinberg, R. A., Cheifetz, S., and Massagué, J. (1988). Science *240*, 196–199.

Kondo, M., Tashiro, K., Fujii, G., Asano, M., Miyoshi, R., Yamada, R., Muramatsu, M., and Shiokawa, K. (1991). Biochem. Biophys. Res. Commun. *181*, 684–690.

Laiho, M., DeCaprio, J. A., Ludlow, J. W., Livingston, D. M., and Massagué, J. (1990). Cell *62*, 175–185.

Laiho, M., Weis, F. M. B., Boyd, F. T., Ignotz, R. A., and Massagué, J. (1991). J. Biol. Chem. *266*, 9108–9112.

Lin, H. Y., Wang, X.-F., Ng-Eaton, E., Weinberg, R. A., and Lodish, H. F. (1992). Cell *68*, 775–785.

López-Casillas, F., Cheifetz, S., Doody, J., Andres, J. L., Lane, W., and Massagué, J. (1991). Cell *67*, 785–795.

Mathews, L. S., and Vale, W. (1991). Cell *65*, 973–982.

Mathews, L. S., Vale, W. W., and Kintner, C. R. (1992). Science *255*, 1702–1705.

Moses, H. L., Yang, E. Y., and Pietenpol, J. A. (1990). Cell *63*, 245–247.

Pietenpol, J. A., Stein, R. W., Moran, E., Yaciuk, P., Schlegel, R., Lyons, R. M., Pittelkow, R. M., Münger, K., Howley, P. M., and Moses, H. L. (1990). Cell *61*, 777–785.

Roberts, A. B., and Sporn, M. B. (1990). In Peptide Growth Factors and Their Receptors, M. B. Sporn and A. B. Roberts, eds. (Heidelberg, Germany: Springer-Verlag), pp. 419–472.

Segarini, P. R., and Seyedin, S. M. (1988). J. Biol. Chem. *263*, 8366–8370.

Ullrich, A., and Schlessinger, J. (1990). Cell *61*, 203–212.

Wang, X.-F., Lin, H. Y., Ng-Eaton, E., Downward, J., Lodish, H. F., and Weinberg, R. A. (1991). Cell *67*, 797–805.

Weinberg, R. A. (1991). Science *254*, 1138–1146.

**So Yc
Neurc**

**Culturir
Edited
Cambric
$45.00.**

As rese
too has
The adv
and con
ogists t
neurites
For exa
Montalc
tured ch
growth
neurotrc
late neu
Other re
neurons
cific syn
extracel
This str
involved
produce
Two
swered
synthesi
lates wi
colleagu
ture to d
pable of
found th
on its ne
Some of
others a
outgrow
biologist
ies and
ties of a
to be, cl
tems, in
bined th
choline
accessit
same cu
from the
genesis.
Despit
there are
far, ther
from ma